SEARCHING FOR IMMUNE TOLERANCE MANIPULATING NEW MOLECULES AND EXPLOITING NEW CONCEPTS ON LYMPHOCYTE BIOLOGY

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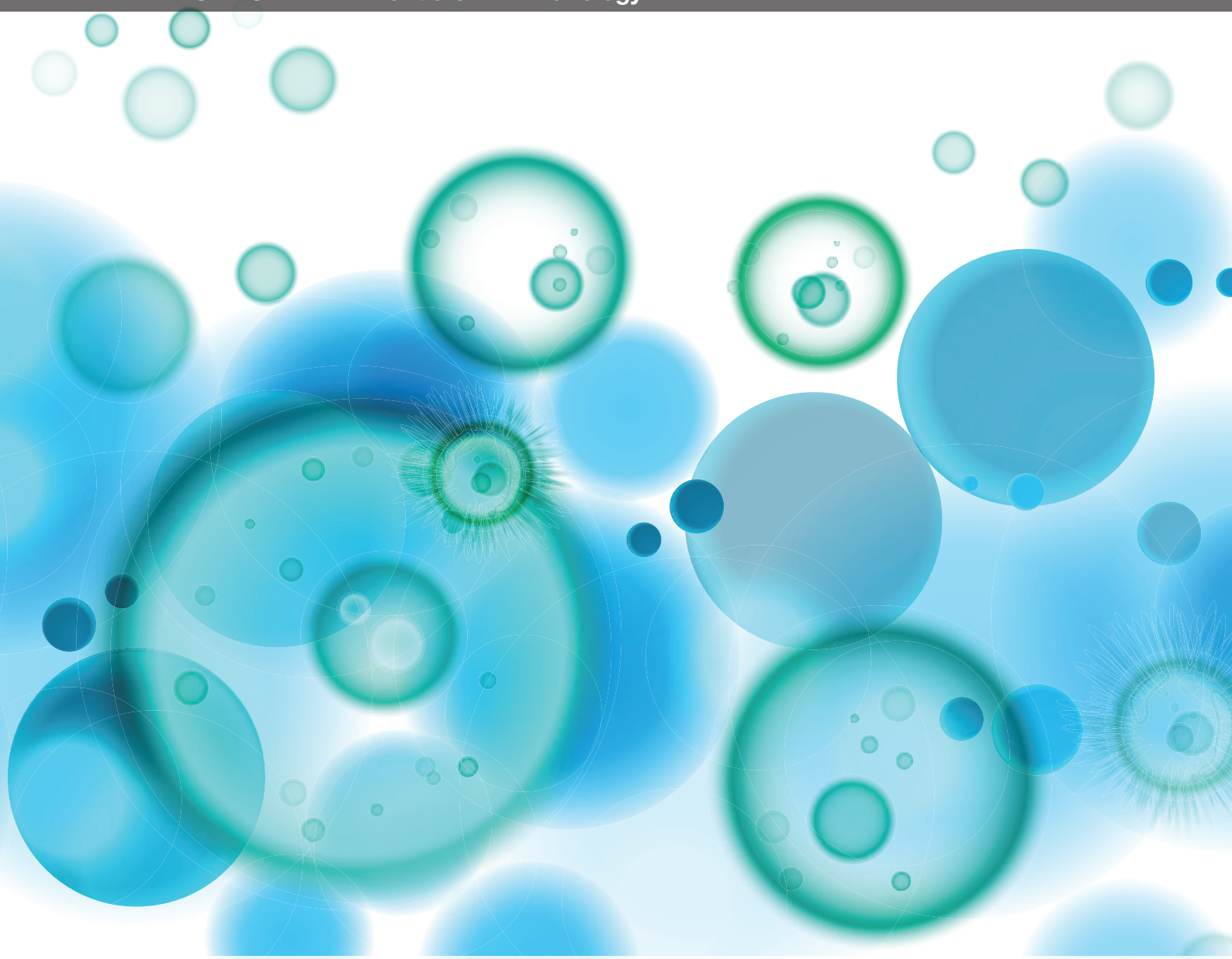

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SEARCHING FOR IMMUNE TOLERANCE MANIPULATING NEW MOLECULES AND EXPLOITING NEW CONCEPTS ON LYMPHOCYTE BIOLOGY

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The break on immune tolerance is a common point between autoimmune diseases and the uncontrolled effector immune responses against allo-antigens in transplantation. Among the past years, several approaches to restore a suppressive immune state have included the targeting of co-stimulatory/inhibitory molecules on immune cells, the promotion or blockade of pivotal cytokines, and the extensive study on how to isolate and expand suppressive cells with the purpose to re-infuse them in patients. To date, the availability of new technologies has permitted to learn, in a more detailed way, the immune mechanisms carried out by suppressive lymphocytes, together with the identification of new potential candidates to target in our quest for immune tolerance. For example, the attractive concepts of lymphocyte plasticity and function stability, supported by the finding of new transcription factors, have opened a new window in the understanding of T cell differentiation, effector cell commitment and immune regulatory function. On the other hand, the discovery of new members of the Ig superfamily ligand, VISTA; the intriguing role of modulatory molecules like Retinoic Acid, Neuropilin-1, Fc gamma receptors, or cytokines such as IL-33, among others, are revealing new possibilities in the development of new strategies to conquer our obsession: immune tolerance. Here, we gather the latest information regarding new targets and cellular processes, including an update on current cellular therapies and the exciting coming approaches to cure autoimmunity and permit transplant acceptance.

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Table of Contents

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EDITORIAL
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Editorial: Searching for Immune Tolerance Manipulating New Molecules and Exploiting New Concepts on Lymphocyte Biology

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The Editorial on the Research Topic

Searching for Immune Tolerance Manipulating New Molecules and Exploiting New Concepts on Lymphocyte Biology

This research topic was inspired in those still non-curable inflammatory conditions, such as autoimmune diseases and transplant rejection, based on the fact that immunologists worldwide are still searching for strategies to restore long-term immune tolerance. Thus, we gathered several researchers whom actively base their investigation lines in novel molecules and immune cell populations that can be exploited to design new strategies for the establishment or recovery of tolerance.

In the context of autoimmunity, intriguing is the role of interferon (IFN)-γ in the pathogenesis of multiple sclerosis (MS) and its animal model, the experimental autoimmune encephalomyelitis, which is reviewed in two articles contained in this edition (Ottum et al.; Arellano et al.). They focus on new evidence that help to explain the seemingly opposing effects of this cytokine over different central nervous system cells and on different stages of the disease, giving some important clues that can help to guide the potential therapeutic use of IFN-γ in MS patients. Other cytokine that recently has been focus of interest is IL-33, a molecule first described as an alarmin, but Gajardo-Carrasco et al. detail the plethora of now recognized functions in which IL-33 is involved, with special attention in T cell biology, adaptive immunity, tolerance, and immunological disorders.

Continuing with an update on molecules with pivotal immune function, Le Mercier et al. and Guo and Wang deliver us a solid snap shot on receptors and ligands with stimulatory and inhibitory immune activity, revising both classic and newest members, their contribution to disease and how they have been currently targeted to utilize them for therapeutic purposes. Special attention received the new Ig family member VISTA, which is presented as an interesting modulator of the immune response and with high potential for its exploitation in the clinic.

Similarly, the article by Iruretagoyena et al. addresses the immune regulatory aspects of vitamin D and its importance in controlling the development of autoimmune diseases. This review has a particular emphasis on the participation of this vitamin in the physiopathology of systemic lupus erythematosus (SLE) and gives an update on the latest data about vitamin D supplementation in SLE patients.

Regarding the use of immune cells with therapeutic purposes, this research topic contains five reviews that put the spotlight over the use of dendritic cells (DCs) and regulatory T cells (Tregs) as tools to treat immune-related conditions (including autoimmunity and transplant rejection). The

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article by Schinnerling et al. summarizes the recent advances in the description of intracellular pathways and transcriptional regulators that command the monocyte-derived tolerogenic human DCs differentiation program and propose candidate molecules that could be regarded as key in their tolerogenic functions. On the other hand, Maggi et al. examine one of the putative mechanisms of action of tolerogenic DCs; this is the induction of hyporesponsive or anergic $CD4^+$ T cells. The authors review recent findings in the impact of $CD4^+$ T cells anergy induction in animal models of autoimmune diseases development and progression, and discuss on the potential benefits of exploiting this mechanism for therapeutic purposes in humans. Similarly, Osorio et al. present a complete revision on DCs nature, from their origin, lineages, differentiation process, subtypes, and physiological role, linking these observations with diseases and mentioning current technological approaches to use them as a source for cellular therapy. On the other hand, Safinia et al. and Gregori et al. targeted human Tregs, describing extensively all their phenotypic characteristics, the different subpopulations identified to date based on certain surface markers and their mechanisms to drive immune suppression, and compiling simultaneously all the results from finished and ongoing clinical trials. In addition, both works discuss different aspects of human Tregs clinical grade manufacture and the variables that need to be improved to perfect the protocol, such as viability, antigen-specificity, cell expansion efficiency, and phenotypic/functional stability.

In parallel, the original article by Ruiz et al. proposes a modified protocol to favor mixed chimerism and further transplant acceptance in a preclinical model. Their novelty bases in the use of antigen-specific Tregs generated *in vitro* in the presence of IL-2, TGF-β, and retinoic acid (RA), in conjunction with previously established procedures as non-myeloablative irradiation and administration of immunosuppressant drugs. This group observed that the transfer of RA-Tregs facilitates donor-cells engraftment and allows for the acceptance of skin allografts, proposing the inclusion of Tregs as co-therapeutic tool. Another article reports how Soto et al. pinpoint at another cell population, frequently overlooked when it comes to tolerance mechanisms: B cells. Using systemic sclerosis (SSc) as a paradigmatic autoimmune disease with cellular and humoral components, the authors describe alterations in the expression levels of activator and inhibitor receptors on B cells from SSc patients that could contribute with the hyperactivated phenotype of these cells. They also demonstrate that IL-10-producing B cells and IL-10 secretion by stimulated B cells are reduced in SSc patients, which can imply that these patients have an impaired anti-inflammatory function on regulatory B cells, a subset specifically dedicated to promote tolerance to innocuous antigens. The restoration of the capacity of these cells to express adequate levels of protolerogenic molecules and regain their regulatory capability through novel or current B cell-targeted therapies could be a promising therapy for SSc or related autoimmune diseases.

Finally, the review by Parigi et al. brings us to a different face of immune tolerance, the one that keep us from mounting exacerbated immune responses against food antigens and commensal microbiota. Disruptions of the tolerogenic mechanisms displayed by a normal intestinal immune system can lead to severe conditions, such as food allergy or inflammatory bowel diseases. This review deals with the way how diet, breast milk, and solid food shape the immune system of newborns and defines the homeostasis in the intestinal microenvironment, thus conferring risk or protection for the future development of immune mediated diseases.

Overall, we achieved putting together a nice compilation on the current molecules and cell populations that are being aggressively targeted to restore immune tolerance in diseased patients. While many efforts are put in translational immunology, basic science immunologists continue working to satisfy these goals.

AUTHOR CONTRIBUTIONS

DC wrote the editorial for this research topic. KP-L coordinated this research topic and wrote the editorial.

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REVIEW
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Novel immune check-point regulators in tolerance maintenance

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The great success of anti-cytotoxic lymphocyte antigen 4 (CTLA4) and anti-programed cell death protein 1 (PD1) in cancer treatment has encouraged more effort in harnessing the immune response through immunomodulatory molecules in various diseases. The immunoglobulin (Ig) super family comprises the majority of immunomodulatory molecules. Discovery of novel Ig super family members has brought novel insights into the function of different immune cells in tolerance maintenance. In this review, we discuss the function of newly identified B7 family molecules, B7-H4 and V-domain Ig Suppressor of T cell Activation (VISTA), and the butyrophilin/butyrophilin-like family members. We discuss the current stages of immunomodulatory molecules in clinical trials of organ transplantation. The potential of engaging the novel Ig superfamily members in tolerance maintenance is also discussed. We conclude with the challenges remaining to manipulate these molecules in the immune response.

Keywords: B7, butyrophilin, immune tolerance

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Introduction

The immune system consists of various cell types to mount both innate and adaptive immunity against pathogens. As an important component of adaptive immunity, T cell activation requires three signals. Signal 1 is acquired through the interaction between T cell receptor (TCR) and peptide-loaded major histocompatibility complex (MHC) molecules. Signal 2 is induced through the interaction between co-stimulatory/co-inhibitory molecules on antigen-presenting cells (APC) and their receptors on T cells, thus enhancing/inhibiting T cell activation. Signal 3 is mediated by cytokines, such as IL2 to promote T cell proliferation. Co-stimulatory/co-inhibitory molecules are the most well-studied molecules and fall into immunoglobulin (Ig) super family members, including B7 family and tumor necrosis factor (TNF) family proteins. These molecules play essential roles in maintaining the balance between sufficient immune response against pathogenic antigens (Ag) as well as limited response against self-antigens or allogenic transplant graft. We identify this family of molecules as "immune modulatory molecules." The potential receptors and ligands for many novel molecules remain unknown and may serve as both receptors and ligands in different cells. In this review, we define the potential receptors/ligands of these novel molecules as "binding partners" for simplicity.

Over the past decade, a large number of B7 and TNF family molecules have been discovered and actively evaluated in clinic. The clinical success of anti-CTLA4 (1, 2) and anti-PD1 blocking antibody (Ab) (3, 4) in various cancers encourages more active research and drug development effort. This effort aims to enhance co-stimulatory or dampening co-inhibitory molecules effect to achieve robust anti-tumor immune response. In parallel, studies are also carried out to dampen the immune response by enhancing the effect of co-inhibitory molecules in treatment of autoimmune or inflammatory diseases (5–7). In addition, organ transplantation is still a great challenge in

the clinic as the allogenic transplant rejection is a common problem in patients. There is great need for inhibiting the host rejection of donor organs (8). Undoubtedly, the discovery of new immune modulatory molecules has offered new opportunities to manipulate the host immune system for tolerance maintenance and graft acceptance. In this article, we review recent discoveries of novel Ig B7 family members VISTA and B7-H4. We also review the butyrophilin (BTN) family members, namely "old molecules with novel immunomodulatory functions." Learning from the ongoing clinical trials on well-described molecules, we discuss the potential application of these new molecules.

B7 Family

B7 family proteins are among the best-described immunomodulatory molecules so far. This family consists of both co-inhibitory and co-stimulatory molecules. This family includes B7.1 (CD80), B7.2 (CD86), CD28, CTLA4, PD1, PD1 ligand (PDL1), PDL2, B7RP1 (ICOSL), B7-H3, B7-H4, and VISTA. The growing list of B7 family members offers great opportunities to modulate immune response therapeutically in treating different diseases. This family of molecules was reviewed elsewhere thoroughly (9, 10). In the following section, we will discuss the progress in understanding two new molecules in the family, B7-H4 and VISTA.

B7-H4

B7-H4 (B7x, B7S1, VTCN1), identified in 2003 (11), is a highly evolutionarily conserved molecule that shares 87% amino acid identity between human and mouse. Mature B7-H4 is a 50–80 kDa transmembrane protein with one IgV domain and one IgC domain. The mRNA expression of B7-H4 is found on both lymphoid and non-lymphoid tissues. However, B7-H4 protein expression is more restricted and not expressed on *ex vivo* human immune cells (T, B, DC, and monocytes), but can be induced after *in vitro* stimulation. Endocrine cells in pancreas have also been shown to express a moderate level of B7-H4. Signaling through B7-H4 pathway leads to the inhibition of TCR-mediated $CD4^+$ and $CD8^+$ T cell proliferation, cell-cycle progression, and IL-2 production (11–13). In experimental autoimmune encephalomyelitis (EAE) model, blocking B7-H4 on host cells by a monoclonal antibody accelerates T cell responses and enhances disease severity (11). However, B7-H4-deficient mice only mount mildly increased Th1 response and intact cytotoxic T-lymphocyte reactions against viral infections (14). The study indicated that B7-H4 is a negative regulator of T cell activation but only plays a minor role in fine-tuning T cell immunity. However, elevated soluble form of B7-H4 in the serum of rheumatoid arthritis (RA) patients is associated with increased disease severity. In a collagen-induced arthritis mouse model, both overexpression of soluble B7-H4 and genetic deletion of B7-H4 enhanced disease severity, due to enhanced B cell, T cell, and neutrophil response (15). This severity suggests that soluble B7-H4 acts as a decoy molecule to block the inhibitory function of B7-H4. Further confirmation of the correlation between soluble B7-H4 and disease severity in RA patients may indicate a new target pathway in autoimmune diseases. Although soluble B7-H4-immunoglobulin (B7-H4-Ig) fusion protein binds to activated T cells, the binding partner for B7-H4 has not been identified and does not bind to known CD28 family members, such as CD28, CTLA4, ICOS, and PD1.

On the other hand, overexpression of B7-H4 on the pancreatic islets can protect mice from T cell-mediated autoimmunity and these islet allograft increased survival after transplantation (16). The immunosuppressive function of B7-H4 has also been investigated in experimental type I diabetes models using B7-H4-Ig. Pre-diabetic non-obese diabetes mice treated with B7-H4-Ig displayed lower incidence of diabetes by suppressing immune infiltrates into the islets (17). Lee et al. found that B7-H4-Ig treatment decreased Th17 cell whereas promoting $CD4^+IFN\gamma^+$ T cells development (18). These findings suggest a possible role of B7-H4 in maintaining the balance between Th1 and Th17 cells. However, more studies are warranted to verify this observation and elucidate the molecular mechanism. The impact of B7-H4 on T cells is summarized in **Figure 1**.

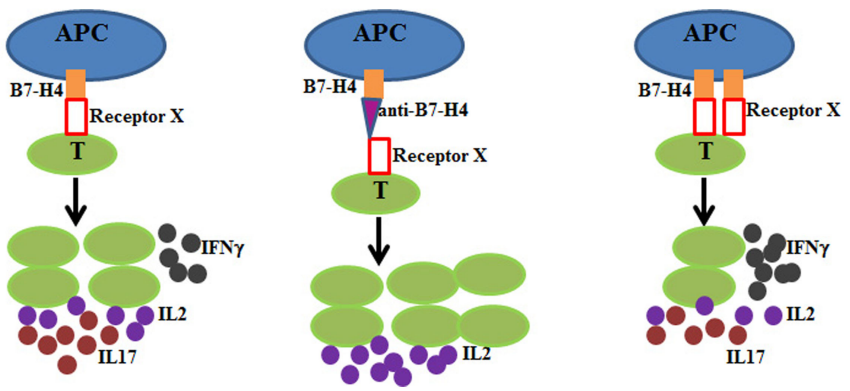

FIGURE 1 | B7-H4 is a co-inhibitory molecule on APC (blue) and interacts with its unknown receptor (receptor X) on T cells (green) to deliver negative signaling during T cell activation. Left: in the presence of B7-H4-receptor X interaction, T cells proliferate at a lower rate and produce lower amount of IL2 (purple), lower IFNγ (black), and higher IL17 (red). Middle: antagonistic anti-B7-H4 (magenta triangle) blocks the interaction between B7-H4 and receptor X. T cells proliferate at a higher rate and produce more IL2. Right: B7-H4 overexpression leads to lower T cell proliferation, lower IL2 and IL17 production, and higher IFNγ production.

In addition to the direct impact of B7-H4 inhibition on T and B cell immune response, B7-H4 also acts as a negative regulator for neutrophil response. B7-H4-deficient mice are more resistant to infection by *Listeria monocytogenes* due to augmented neutrophil accumulation (19). Within 3 days after infection, bacterial colonies in liver and spleen are significantly lower in B7-H4-deficient mice than controls, concomitant with increased number of neutrophils in the spleen of infected mice. B7-H4 inhibits the growth of bone marrow-derived neutrophil progenitors *in vitro*, demonstrating an inhibitory function of B7-H4 in neutrophil expansion. Enhanced neutrophil growth in B7-H4-deficient mice also occurs in the background of recombination activation gene (RAG) knock out mice, independent of adaptive immunity. The inhibition of neutrophil expansion shows that B7-H4 directly regulates innate immunity. Furthermore, Pawar et al. discovered B7-H4 expression on kidney resident cells and could be induced further with inflammatory stimuli. In an antibody-mediated nephritis model, B7-H4-deficient mice showed more T, B, macrophage, and neutrophil infiltration into kidney. In addition, macrophages in B7-H4-deficient mice demonstrate a more inflammatory phenotype. Whether B7-H4 modulates other immune cells remains unknown.

V Domain-Containing Ig Suppressor of T-Cell Activation

V domain-containing Ig suppressor of T-cell activation (VISTA), also named as PD1 homolog (PD1H), is expressed in both hematopoietic and non-hematopoietic tissues. Among immune cells, VISTA is highly expressed on mature $CD11b^{high}$ myeloid-derived APCs and to a less extent on $CD4^+$, $CD8^+$, and Tregs (20). VISTA is a 55- to 65-kDa type I Ig membrane protein with the extracellular domain homologous to PD-L1. *In vitro*, VISTA-Ig inhibits T cell activation, proliferation, and production of cytokines, such as IL2 and IFNγ, during anti-CD3 activation. VISTA overexpression on APC also suppressed T cell proliferation, which was rescued by neutralizing anti-VISTA monoclonal Ab (13F3). More importantly, VISTA overexpression on tumor cells overcame the protective anti-tumor immunity. These studies collectively suggest that VISTA on APC delivers negative signaling to T cells during activation via a yet-to-identify binding partner on T cells. The same Ab administration *in vivo* exacerbated disease progression in a passive EAE model. Further studies demonstrated that mice deficient in VISTA display normal hematopoietic development. These mice do not succumb to obvious organ-specific autoimmune diseases, despite chronic inflammation in multiple organs over age. Consistently, spontaneously activated T cells accumulate and produce inflammatory cytokines and chemokines in aged VISTA-deficient mice (21). 2D2 T cell receptor mice overexpress T cell receptor that recognizes myelin oligodendrocyte glycoprotein (MOG_{35-55}). VISTA deletion in 2D2 transgenic mice significantly enhanced EAE severity. Disease progression is due to an increase in activated encephalitogenic T cells in the periphery and greater infiltration into the central nervous system. The analysis of both VISTA-Ig and genetic ablation demonstrated that VISTA is a negative check-point regulator of T cell activation. Blocking VISTA's function promotes the pro-inflammatory cytokines production and enhances autoimmune diseases development under susceptible conditions. However, another anti-VISTA Ab seemed to inhibit the progression of acute graft-versus-host-diseases (GVHD). This inhibition indicates the agonistic effect of this Ab in the regard of suppressing T cell activation (22).

Even though the binding partner of VISTA remains unknown, Flies et al. demonstrated that VISTA is a co-inhibitory receptor on $CD4^+$ T cells. $VISTA^{-/-}$ $CD4^+$ T cells showed stronger Ag-specific proliferation and cytokine production than wild-type $CD4^+$ T cells. In an APC-T co-culture study, VISTA deficiency on both APC and T cells resulted in the strongest proliferation response, whereas VISTA-sufficient APC and T cells generated the poorest response. VISTA deficiency on either APC or T cells yielded an intermediate response. Taken this data together with previous studies (20), VISTA negatively regulates T cell immunity via direct impact on T cells by engaging different receptor/ligand (illustrated in **Figure 2**). In a murine model of acute hepatitis, a different agonistic VISTA Ab administration suppressed $CD4^+$ T cell and NK cell-mediated acute inflammation. The data suggested that $VISTA^{-/-}$ mice showed survival benefits during GL261 glioma growth, mediated by enhanced $CD4^+$ T cell anti-tumor immunity (22). The bidirectional inhibition of VISTA on APC and T cells suggests that VISTA is a valuable target for immunological diseases. The studies in mice are reinforced by characterization of human VISTA expression and function (23). More studies are needed to characterize the correlation between VISTA expression and autoimmune diseases, such as RA, to establish the rationale for targeting VISTA in clinic. Moreover, it is worthwhile evaluating the potential of agonistic anti-VISTA Ab in tolerance induction during organ transplantation given the preclinical evidences in GVHD. Furthermore, the identification of VISTA's binding partner will certainly offer great insight into the molecular mechanism of VISTA signaling and ensure better understanding of agonistic Ab safety in development.

Butyrophilin Family in Immune Tolerance

As B7 family member proteins, BTN family members have extracellular IgV and IgC domain, but most BTN family members have an intracellular B30.2 domain. Therefore, BTN family members belong to the Ig superfamily. Since the discovery of BTN family

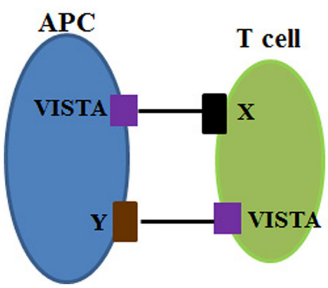

FIGURE 2 | VISTA is a co-inhibitory molecule on both APC (blue) and T cells (green). VISTA is expressed on APC and interacts with the partner (receptor X) on T cells. VISTA can also be a co-inhibitory receptor on T cells and binds to its unknown ligand (ligand Y) on APC. Both interactions deliver negative signaling to T cell activation.

members in the regulation of immune response, butyrophilin-like (BTNL), family members have also been identified in both human and mouse genome. The extracellular structural similarity between BTN/BTNL and B7 family suggested that BTN/BTNL family molecules may have immune regulatory functions as B7 family members. In this section, we discuss the genomic distribution, function, and clinical evidence between BTN/BTNL family proteins and immune tolerance.

Genomic Distribution of BTNs

The first BTN member was discovered in milk in which BTN subfamily 1 member A1 (BTN1A1) is essential for the secretion, lactation, and stabilization of milk-fat globules (24–26). Further studies discovered more BTN proteins with immune-regulatory functions. Thus far, 13 human BTNs and BTNLs have been described. Human BTNs include BTN1A1, BTN2A1, BTN2A2, BTN2A3, BTN3A1, BTN3A2, and BTN3A3 (27). Human BTNLs include BTNL2, BTNL3, BTNL8, BTNL9, BTNL10, and SKINT-like (SKINTL). In mice, 11 genes have been identified to encode BTN and BTNL molecules. These include BTN1A1, BTN2A2, BTNL1, BTNL2, BTNL4, BTNL5, BTNL6, BTNL7, BTNL9, BTNL10, and SKINT. The majority of these proteins are diverse between human and mice, making it difficult to study their function. As mentioned above, the BTN family members have extracellular IgV and IgC domains as B7 family proteins (28–31). Extracellular IgV and IgC domains are essential for the interaction among B7 family members. However, it remains unknown whether BTN/BTNL family proteins bind to their counter-receptors via the IgV and IgC domain. In addition, it remains unknown how intracellular B30.2 domain binds to other intracellular signaling proteins to deliver the function of BTNs and BTNLs. Regarding the expression pattern of BTN/BTNL family molecules, there is some inconsistency among existing data when comparing mRNA with protein expression. Abeler-Dorner et al. summarized the mRNA and protein expression of each BTN/BTNL family member in another review (27).

Function of Butyrophilin in T cell Immunity

The wide distributions of certain BTN/BTNLs determine the functions of these molecules in different immune cells. As BTN/BTNLs are expressed on APCs or T cells, studies have been carried out to determine the function of these molecules in the T cell immunity *in vitro* and *in vivo*. In this section, we will focus on the function of these "old molecules" in regulation of T cell immunity, in the regard of both conventional αβ T cells and γδ T cells.

Butyrophilin in Regulation of Conventional T Cell Response

As B7 family members, some BTN/BTNLs are co-inhibitory whereas others are co-stimulatory in the regard of conventional T cell function regulation. The function characterization of BTN/BTNL family members depends on the administration of recombinant protein and antagonistic/agonistic Ab in T cell activation, differentiation, and cytokine production *in vitro* and diseases progression *in vivo*. In the following section, we will focus on the delineation of co-inhibitory and co-stimulatory function of various BTN/BTNL family members.

BTNs as co-inhibitory molecules

Nguyen et al. first demonstrated the co-inhibitory role of mouse BTNL2 in T cell immunity (32). Mouse BTNL2-Ig fusion protein inhibited anti-CD3- and anti-CD28-stimulated $CD4^+$ T cell proliferation and IL2 production via reducing activating protein-1 (AP-1), nuclear factor of activating T cells (NFAT), and NF-κB activity. Arnett et al. further illustrated the upregulation of BTNL2 in inflamed colons in the mouse Mdr1a-deficient model of inflammatory bowel diseases. BTNL2 conveys its inhibitory signal also by counteracting the co-stimulatory effect of B7RP1 (ICOS ligand) on T cell activation (33). No binding partner has been successfully identified as BTNL2 failed to bind known B7 family proteins, such as CD28, ICOS, PD1, CTLA4, and HVEM.

Further studies elucidated the similar co-inhibitory function of murine BTNL1 (34). *In vitro* studies showed that BTNL1-Ig fusion protein suppressed low-concentration anti-CD3-induced T cell activation and IL2 production by cell-cycle arrest. Like BTNL2, BTNL1 does not bind CD28, CTLA4, triggering receptor expressed on myeloid cells (TREM)-like transcript 2 (TLT-2, TREML2), B7-H3, B7-1, or other B7 family members. Antagonistic anti-BTNL1 Ab-induced strong Th2 response after keyhole limpet hemocyanin (KLH) and complete Freund's adjuvant (CFA) immunization *in vivo*, demonstrated by enhanced T cell proliferation and higher production of Th2 cytokines (IL-4, IL-5, and IL-13), but lower IFNγ. Antagonistic anti-BTNL1 Ab treatment also exacerbated EAE via promoting Th17 response. Similarly, α-BTNL1 Ab exacerbated Th2-mediated allergy response. These studies indicate that BTNL1 may fine tune Th1 versus Th2 and Th17 development. No potential BTNL1 binding partner has been identified so far.

Human BTN3A1, also known as CD277, is highly upregulated in myeloid DCs and macrophages by vascular endothelial growth factor (VEGF) and CCL3 in human ovarian cancer. BTN3A1 inhibited T cell proliferation and Th1 cytokine production through preventing the upregulation of cellular caspase-8 (FLICE)-like inhibitory protein (cFLIP) and the consequently enhanced caspase-8 activity. Caspase-8 activity is necessary to initiate the activation of NF-κB and promote T cell proliferation (35). A recent study, however, extended the function of BTN3A1 beyond the co-inhibition to presenting phosphorylated antigen (discussed in more details below).

Even though the preclinical studies highlighted the importance of these co-inhibitory BTN/BTNLs in inflammatory diseases and possibly graft tolerance, key issues remain for therapeutic targeting of these molecules. First, little is known regarding how the negative signaling to T cell activation is conveyed by the engagement with their potential binding partners. The understanding in this regard will facilitate the identification of pharmacological kinetics (PK) and pharmacological dynamics (PD) markers during therapeutic Ab screening. Second, no potential binding partners for these molecules have been identified. To enhance the negative effect of the molecules during APC–T cell interaction, it is essential to minimize unwanted effects by activating potentially complex signaling pathway in T cells. More studies are needed to gain better understanding of the function of these co-inhibitory molecules in various T cell subsets differentiation and other immune cells. This will help to determine the specific need

of the Ab isotype or the format of BTN/BTNL-Ig fusion protein to deliver the most effective drug with least toxicity.

BTNs as co-stimulatory molecules

Among BTN/BTNL family members, BTNL8 and BTN3A2 are co-stimulatory molecules. There is no ortholog of human BTNL8 in mice. BTNL8 mRNA is highly expressed on neutrophils rather than T cells or myeloid cells. BTNL8 seems to bind resting instead of activated T cells. The administration of BTNL8-Ig enhanced anti-CD3-stimulated human $CD4^+$ T cell proliferation and cytokines production, such as IFNγ, TNFα, IL8, and IL10. This study indicated that BTNL8-Ig functions at very early stage of T cell activation (36). Surprisingly, BTNL8-Ig fusion protein enhanced Ovalbumin and alum-stimulated primary Ab response in mice. However, no studies have illustrated the reason for cross-species activity of BTNL8-Ig. Therefore, the binding partner of human BTNL8 may share high homology with its ortholog in mice. BTNL3 shares high sequence similarity BTNL8 but is expressed mainly in neutrophils. No known function of BTNL3 has been reported. In a population genetics study, Aginel et al. identified a *BTNL8–BTNL3* deletion leading to a new BTNL8*3 fusion protein (37). The sequence between intron 4 of BTNL8 and intron 4 of BTNL3 is deleted, leading to no coding sequence changes in BTNL8–BTNL3. Thus, the fusion protein may still be intact. Gene-expression analysis in lymphoblastoid cell lines showed that *BTNL8–BTNL3* deletion is correlated with TNF and ERK1/AKT pathways, the key players in immune regulation. However, function analysis needs to be done. Further studies are warranted to elucidate the correlation between *BTNL8–BTNL3* deletion and inflammatory diseases in patients.

BTN3A2 was recognized as a co-stimulatory molecule from correlation studies in cancer patients. Earlier studies suggested that high BTN3A2 mRNA expression was associated with a good prognosis in relation to disease-free and overall survival in a cohort of 55 epithelial ovarian cancer (EOC) patients (38). Further studies in a larger cohort of 199 high-grade EOC patients confirmed that the protein expression of BTN3A2 in ovarian cancer tissues is positively correlated with the intraepithelial infiltration of $CD4^+$ and $CD8^+$ T cells (39). The validation of BTN3A2 as a prognostic marker for EOC indicated that BTN3A2 may modulate the infiltration of immune cells and thus the anti-cancer immunity. To better understand whether BTN3A2 can be a therapeutic target in cancer, inflammatory diseases, or transplant tolerance, the function of BTN3A2 in specific immune cells warrants further studies.

BTN as Antigen Presentation Molecule

A very recent study demonstrated the function of BTN3A1 beyond the co-inhibitory and co-stimulatory roles in immune modulation (40, 41). Human Vγ9Vδ2 T cells are activated by phosphorylated antigens. Theses antigens include host isopentenylpyrophosphate (IPP) and microbial (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP). Vavassori et al. discovered that recombinant BTN3A1 specifically stimulated human Vγ9Vδ2 T cells in the presence of IPP. This effect is independent of CD1d molecule. BTN3A1 binds to IPP at a stoichiometry ratio of 1:1 at a lower affinity ($K_d = 10^{-6}$ M) than the binding of MHC to peptides ($K_d = 10^{-7} \sim 10^{-9}$ M). However, phosphorylated antigens can be pulsed on APCs, indicating that they are bound by BTN3A1 under assay and physiological conditions. Direct binding between BTN3A1 and Vγ9Vδ2 T cells was specific and weak in the absence of IPP. However, BTN3A1 and Vγ9Vδ2 T cells binding is facilitated by IPP engagement. This new role of BTN3A1 is far beyond co-stimulatory molecules in conventional αβ T cells as reported previously (35). Human Vγ9Vδ2 T cells are critical components of protective anti-microbial and anti-cancer immunity that involve cells that upregulate IPP. Therefore, it is of great value to understand how BTN3A1 modulates Vγ9Vδ2 cells in inflammatory diseases and cancer patients. Therapeutic effect of targeting BTN3A1 may depend on the function of Vγ9Vδ2 in specific diseases. Therapeutic targeting of BTN3A1 may potentially expand the bandwidth of immune regulation in clinic.

Function of BTN/BTNL in APC

In addition to co-inhibitory function of BTNL1 T cell immunity, a recent study focused on the function in enterocytes (42). BTNL1 is highly expressed in small intestine, particularly on all enterocytes in close juxtaposition with T cells. When an enterocyte-derived cell line is transfected with *Btnl1*, $CD8\alpha\beta^+TCR\alpha\beta^+$ and $CD8\alpha\alpha^+\gamma\delta$ intraepithelial lymphocytes-induced cytokine production was inhibited in a cell–cell contact-independent manner. The cytokines include IL6, CXCL1, CCL4, and IL15. However, monocyte chemoattractant protein-1 (MCP1), IFNγ, and CCL2 remained unchanged by *Btnl1* overexpression. As epithelial cells are protective sites where infection occurs frequently, the capability of BTNL1 to dampen the response of enterocytes to activated intraepithelial lymphocytes may be essential for leveraging colitis and colon cancer. The close juxtaposition between BTNL1 and cell receptor suggested that the interaction between intraepithelial lymphocytes and enterocytes plays an essential role in sustaining the integrity of colon tissues. Nevertheless, it remains unknown whether BTNL1 controls the development of colon inflammation *in vivo* or not. More *in vivo* studies using the specific deletion or overexpression of BTNL1 in enterocytes may facilitate the understanding and therapeutic targeting of BTNL1 in inflammatory diseases and tolerance induction.

Clinical Indication of BTN/BTNL and Immune Tolerance

The first clinical association between BTN/BTNL proteins and immune function modulation was identified between BTNL2 mutation and sarcoidosis (43). SNP analysis of 947 independent and familial sarcoidosis cases identified a G–A transition constituting rs2076530, leading to a mutant protein lacking the C-terminal IgC domain and transmembrane helix. The mutant protein fails to localize on the cell membrane and loses co-inhibitory function, leading to over-activated T cells and thus overt inflammation. Several other studies reported the increased susceptibility to autoimmune sarcoidosis and myositis in patients bearing BTNL2 mutations (44). In addition, studies also showed correlation between BTNL2 polymorphism and ulcerative colitis (45–47), tuberculosis (48), RA, and systemic lupus erythematosus (49). However, more validation studies are required to establish

the direct correlation between BTNL2 polymorphism and disease progression or severity. Going from bench to bedside, more efforts should be taken to understand the role of different BTN/BTNL family members in modulating the function of specific immune cells in various inflammatory diseases and organ transplantation.

Like some B7 family members, different BTN family members can also interact with unknown binding partners in different cell types, eliciting inhibitory or stimulatory response. Thorough search for the binding partners of these BTN/BTNL members will certainly facilitate the therapeutic development of agonist or antagonist Ab or BTN-BTNL/Ig fusion protein in the organ transplant maintenance. However, as transplant rejection is rather complicated with various immune compartments involved, more preclinical studies are warranted with any potential therapeutic candidate. We will discuss below the current progress of manipulating the well-understood immune modulatory molecules in clinical development and what lessons we can learn from the past.

Transplant Tolerance in Clinical Practice

Transplant tolerance is still challenging in clinical practice, although immunosuppression drugs, such as corticosteroids, have been widely used in transplantation practice. Orlando et al. summarized that out of 461 liver transplantation patients, 163 were successful in maintaining the graft during immunosuppression drugs withdrawal (50). On the contrary, there is only one case report of operational tolerance in lung transplant (51) and one in heart transplant (52).

To meet the clinical needs for tolerance maintenance, different treatments have been developed by depleting effector B and T cells, and enhancing the suppressive capability of Tregs and tolerant DCs. Clinical trials are undergoing through depletion of T cells using a-CD52 (53) and B cell depletion by anti-CD20 (Rituximab) (NCT00568477). Recent studies also explored the efficacy of Treg adoptive transfer (NCT02088931) and bone marrow transplantation (54). To target effector cells and "regulatory" cells simultaneously, some immunomodulatory molecules seem practical based on the current data available in cancer studies. Below, we will discuss specifically the drug development of checkpoint regulators in clinic trial and what this means for future work.

Numerous studies are testing the clinical feasibility of targeting co-stimulatory or co-inhibitory molecules to treat both autoimmune diseases and prolong organ transplant survival. The earliest and most well-understood pathway is CD28/CTLA4–CD80/CD86 pathway. In this regard, CTLA4-Ig fusion protein (abatacept) was the first immune check-point regulator drug approved for the treatment of RA with good safety profile (5, 6). Abatacept binds to both CD80 and CD86, thus inhibiting both co-stimulatory CD28 and co-inhibitory CTLA4 signaling pathway. Early clinical trials of abatacept showed no significant benefit in symptoms of mild atopic asthma (55), multiple sclerosis (56), or ulcerative colitis (57). However, a second generation of CTLA-Ig fusion protein (belatacept) has a lower disassociation rate from CD80 and CD86 (58). In a Phase III trial of renal transplantation, patients treated with belatacept and calcinurin inhibitor showed superior function of renal transplants and multiple other clinical parameters (7, 59). However, belatacept-treated patients also experienced a higher incidence and severity of acute rejection episodes 1 year after transplantation than those patients treated with calcinurin inhibitor only.

Further studies focused on antagonistic CD28 Ab (sc28AT) to block the interaction between CD28 and CD80/CD86 without disturbing the CTLA4 pathway. Non-human primate studies suggested that the combination therapy of sc28AT and calcineurin inhibitor induced CTLA4-dependent decrease of T cell function and Treg promotion in the heart allografts in macaques (60). In addition to the CD28/CTLA4 pathway, the CD40/CD154 pathway has resurged as promising target in the clinic. Non-human primate studies showed that non-depleting CD40 blocking Ab showed efficacy in delaying rejection of bone marrow, islet (61), and renal transplantation. Thus, a Phase IIa clinical trial is underway to measure the efficacy of anti-CD40 (ASKP1240) in renal transplantation (NCT01780844). The clinical trials of targeting these pathways are summarized in **Table 1**.

As we learned from past experiences, different molecules may contribute to various aspects of allograft rejection, through resting, activated, or memory T cell function regulation. Therefore, more effort should be taken to understand the intercrossing signaling pathways mediated by different molecules on APCs or T cells. This will facilitate the identification of the appropriate pathway to target with least side effects. The novel B7 family members and BTN/BTNL members may play different roles than other "old" molecules at different development stages of various cell types. For instance, the high expression of B and T lymphocyte attenuator (BTLA) in γδ T cells (62) may suggest that this molecule is important for modulating the pro-inflammatory activities of γδ T cells. More studies are warranted to determine

TABLE 1 | Summary of the key clinical trials of targeting immune modulatory molecules and specific immune cells to gain immune tolerance.

Test agent	Target	Clinical trial number	Trial phase	Disease area	Reference
Campath-1H (alemtuzumab)	Anti-CD52	NCT00365846	Phase II	Renal transplantation	(53)
Rituximab	Anti-CD20	NCT00568477	Phase II	Renal transplantation	N/A
Treg transfer	Treg	NCT02088931	Phase I	Renal transplantation	N/A
Abatacept	CTLA4-Ig	NCT00048568, NCT00420199	Phase III	Rheumatoid arthritis	(5)
Abatacept	CTLA4-Ig	NCT00784459	Phase II	Mild atopic asthma	(55)
Abatacept	CTLA4-Ig	NCT01116427	Phase I/II	Multiple sclerosis	(56)
Abatacept	CTLA4-Ig	NCT00410410	Phase III	Ulcerative colitis	(57)
Belatacept	CTLA4-Ig	NCT00114777	Phase II	Renal transplantation	(7, 59)
		NCT00346151	Phase III		
ASKP1240	Anti-CD40	NCT01780844	Phase II a	Renal transplantation	N/A

whether co-inhibitory molecules on γδ T cells play a significant role in inflammatory diseases and allograft rejection.

The manipulation of both co-stimulatory and co-inhibitory molecules in the clinic may bring promising outcomes in control of tolerance maintenance. The incoming data from on-going and future clinical trials will shed light on how different co-inhibitory molecules can be engaged to tune down the host response against the donor transplant. More characterization is required to understand the specific impact of these drugs in facilitating Treg generation and inhibiting effector function. The selection of therapeutic Ab isotypes and Ig fusion proteins takes thorough exploration to determine the best therapeutic efficacy with least toxicity. On the other hand, better understanding of the co-stimulatory molecules will facilitate their function analysis in tolerance maintenance during organ transplantation. The antagonistic Ab against the costimulatory molecules may block the effector T and/or B cell function by directly interfering with the ligand–receptor binding on T cells. Alternatively, these molecules can also function via interrogating the suppressive capability of myeloid cells. More studies are needed to address the different functions of these specific molecules in effector and regulatory cells.

Concluding Remarks

The discovery of various new Ig superfamily member proteins has facilitated the clinical treatment of cancer, and tolerance maintenance in inflammatory diseases and organ transplant. The increasing effort on preclinical studies and drug development toward these molecules will eventually determine the best single agent or combinatorial approach with most desirable efficacy and least toxicity. In our view, in-depth understanding of these molecules in the following aspects is critical for clinical benefits. First, in preclinical studies, it is of great importance to identify the unknown binding partners and the down-stream signaling pathways mediated by engagement of both co-inhibitory and co-stimulatory molecules. The identification of binding partners for known molecules, such as VISTA and B7-H4, is essential for understanding the mechanism of antagonistic or agonistic Ab/Ig fusion protein in altering the function of various immune cells. Shared receptor(s)/ligand(s) among different Ig superfamily molecules is not unusual, so the identification of binding partners for new molecules will also help to determine the affected pathways. The intracellular signaling pathway mediated by Ig superfamily proteins engagement is critical for the assay development during Ab or Ig fusion protein screening in drug development. The recently established function of these molecules in inflammatory cells, such as γδ T cells, offers a different angle to target these molecules. Second, clinical studies need to focus on the following aspects to better harness the immune system in tolerance maintenance across different diseases. It is important to establish the correlation between inflammation and BTN/BTNLs, and other novel B7 family members in patients, such as RA, transplant rejection, and so on. From on-going clinical trials, extensive analysis of known immune check-point regulators may shed new light on the optimal target molecules/immune cells. The identification of biomarkers is critical for both the assessment of efficacy and the understanding of mechanism of therapeutic agents. Finally, in inflammatory diseases and organ transplantation treatment, combinatorial approaches targeting Ig superfamily members with current standard of care regimes may potentially offer enhanced therapeutic advantage. However, the complexity of the disease severity and the transplant recipients need to be evaluated carefully before determining the treatment regime due to the potential danger of long-term immunosuppression. In conclusion, more studies will allow for better understanding both the mechanism of these novel molecules in preclinical studies and clinical trials for best development of therapeutic agents in various diseases.

References

1. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* (2011) **364**:2517–26. doi:10.1056/NEJMoa1104621
2. Maio M, Grob JJ, Aamdal S, Bondarenko I, Robert C, Thomas L, et al. Five-year survival rates for treatment-naive patients with advanced melanoma who received ipilimumab plus dacarbazine in a phase III trial. *J Clin Oncol* (2015) **33**:1191–6. doi:10.1200/JCO.2014.56.6018
3. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, et al. Pembrolizumab for the treatment of non-small-cell lung cancer. *N Engl J Med* (2015) **372**:2018–28. doi:10.1056/NEJMoa1501824
4. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus ipilimumab in advanced melanoma. *N Engl J Med* (2015) **372**:2521–32. doi:10.1056/NEJMoa1503093
5. Kremer JM, Westhovens R, Leon M, Di Giorgio E, Alten R, Steinfeld S, et al. Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *N Engl J Med* (2003) **349**:1907–15. doi:10.1056/NEJMoa035075
6. Genovese MC, Becker JC, Schiff M, Luggen M, Sherrer Y, Kremer J, et al. Abatacept for rheumatoid arthritis refractory to tumor necrosis factor alpha inhibition. *N Engl J Med* (2005) **353**:1114–23. doi:10.1056/NEJMoa050524
7. Vincenti F, Charpentier B, Vanrenterghem Y, Rostaing L, Bresnahan B, Darji P, et al. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). *Am J Transplant* (2010) **10**:535–46. doi:10.1111/j.1600-6143.2009.03005.x
8. Chandrasekharan D, Issa F, Wood KJ. Achieving operational tolerance in transplantation: how can lessons from the clinic inform research directions? *Transpl Int* (2013) **26**:576–89. doi:10.1111/tri.12081
9. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol* (2013) **13**:227–42. doi:10.1038/nri3405
10. Ford ML, Adams AB, Pearson TC. Targeting co-stimulatory pathways: transplantation and autoimmunity. *Nat Rev Nephrol* (2014) **10**:14–24. doi:10.1038/nrneph.2013.183
11. Prasad DV, Richards S, Mai XM, Dong C. B7S1, a novel B7 family member that negatively regulates T cell activation. *Immunity* (2003) **18**:863–73. doi:10.1016/S1074-7613(03)00147-X
12. Sica GL, Choi IH, Zhu G, Tamada K, Wang SD, Tamura H, et al. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity* (2003) **18**:849–61. doi:10.1016/S1074-7613(03)00152-3
13. Zang X, Loke P, Kim J, Murphy K, Waitz R, Allison JP. B7x: a widely expressed B7 family member that inhibits T cell activation. *Proc Natl Acad Sci U S A* (2003) **100**:10388–92. doi:10.1073/pnas.1434299100
14. Suh WK, Wang S, Duncan GS, Miyazaki Y, Cates E, Walker T, et al. Generation and characterization of B7-H4/B7S1/B7x-deficient mice. *Mol Cell Biol* (2006) **26**:6403–11. doi:10.1128/MCB.00755-06
15. Azuma T, Zhu G, Xu H, Rietz AC, Drake CG, Matteson EL, et al. Potential role of decoy B7-H4 in the pathogenesis of rheumatoid arthritis: a mouse model

informed by clinical data. *PLoS Med* (2009) **6**:e1000166. doi:10.1371/journal.pmed.1000166
16. Wang X, Hao J, Metzger DL, Ao Z, Meloche M, Verchere CB, et al. B7-H4 pathway in islet transplantation and beta-cell replacement therapies. *J Transplant* (2011) **2011**:418902. doi:10.1155/2011/418902
17. Wang X, Hao J, Metzger DL, Mui A, Ao Z, Akhoundsadegh N, et al. Early treatment of NOD mice with B7-H4 reduces the incidence of autoimmune diabetes. *Diabetes* (2011) **60**:3246–55. doi:10.2337/db11-0375
18. Lee IF, Wang X, Hao J, Akhoundsadegh N, Chen L, Liu L, et al. B7-H4.Ig inhibits the development of type 1 diabetes by regulating Th17 cells in NOD mice. *Cell Immunol* (2013) **282**:1–8. doi:10.1016/j.cellimm.2013.03.005
19. Zhu G, Augustine MM, Azuma T, Luo L, Yao S, Anand S, et al. B7-H4-deficient mice display augmented neutrophil-mediated innate immunity. *Blood* (2009) **113**:1759–67. doi:10.1182/blood-2008-01-133223
20. Wang L, Rubinstein R, Lines JL, Wasiuk A, Ahonen C, Guo Y, et al. VISTA, a novel mouse Ig superfamily ligand that negatively regulates T cell responses. *J Exp Med* (2011) **208**:577–92. doi:10.1084/jem.20100619
21. Wang L, Le Mercier I, Putra J, Chen W, Liu J, Schenk AD, et al. Disruption of the immune-checkpoint VISTA gene imparts a proinflammatory phenotype with predisposition to the development of autoimmunity. *Proc Natl Acad Sci U S A* (2014) **111**:14846–51. doi:10.1073/pnas.1407447111
22. Flies DB, Han X, Higuchi T, Zheng L, Sun J, Ye JJ, et al. Coinhibitory receptor PD-1H preferentially suppresses CD4(+) T cell-mediated immunity. *J Clin Invest* (2014) **124**:1966–75. doi:10.1172/JCI74589
23. Lines JL, Pantazi E, Mak J, Sempere LF, Wang L, O'Connell S, et al. VISTA is an immune checkpoint molecule for human T cells. *Cancer Res* (2014) **74**:1924–32. doi:10.1158/0008-5472.CAN-13-1504
24. Franke WW, Heid HW, Grund C, Winter S, Freudenstein C, Schmid E, et al. Antibodies to the major insoluble milk fat globule membrane-associated protein: specific location in apical regions of lactating epithelial cells. *J Cell Biol* (1981) **89**:485–94. doi:10.1083/jcb.89.3.485
25. Heid HW, Winter S, Bruder G, Keenan TW, Jarasch ED. Butyrophilin, an apical plasma membrane-associated glycoprotein characteristic of lactating mammary glands of diverse species. *Biochim Biophys Acta* (1983) **728**:228–38. doi:10.1016/0005-2736(83)90476-5
26. Stefferl A, Schubart A, Storch M, Amini A, Mather I, Lassmann H, et al. Butyrophilin, a milk protein, modulates the encephalitogenic T cell response to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis. *J Immunol* (2000) **165**:2859–65. doi:10.4049/jimmunol.165.5.2859
27. Abeler-Dorner L, Swamy M, Williams G, Hayday AC, Bas A. Butyrophilins: an emerging family of immune regulators. *Trends Immunol* (2011) **33**:34–41. doi:10.1016/j.it.2011.09.007
28. Tazi-Ahnini R, Henry J, Offer C, Bouissou-Bouchouata C, Mather IH, Pontarotti P. Cloning, localization, and structure of new members of the butyrophilin gene family in the juxta-telomeric region of the major histocompatibility complex. *Immunogenetics* (1997) **47**:55–63. doi:10.1007/s002510050326
29. Ikemizu S, Gilbert RJ, Fennelly JA, Collins AV, Harlos K, Jones EY, et al. Structure and dimerization of a soluble form of B7-1. *Immunity* (2000) **12**:51–60. doi:10.1016/S1074-7613(00)80158-2
30. Arnett HA, Escobar SS, Viney JL. Regulation of costimulation in the era of butyrophilins. *Cytokine* (2009) **46**:370–5. doi:10.1016/j.cyto.2009.03.009
31. Arnett HA, Viney JL. Immune modulation by butyrophilins. *Nat Rev Immunol* (2014) **14**:559–69. doi:10.1038/nri3715
32. Nguyen T, Liu XK, Zhang Y, Dong C. BTNL2, a butyrophilin-like molecule that functions to inhibit T cell activation. *J Immunol* (2006) **176**:7354–60. doi:10.4049/jimmunol.176.12.7354
33. Arnett HA, Escobar SS, Gonzalez-Suarez E, Budelsky AL, Steffen LA, Boiani N, et al. BTNL2, a butyrophilin/B7-like molecule, is a negative costimulatory molecule modulated in intestinal inflammation. *J Immunol* (2007) **178**:1523–33. doi:10.4049/jimmunol.178.3.1523
34. Yamazaki T, Goya I, Graf D, Craig S, Martin-Orozco N, Dong C. A butyrophilin family member critically inhibits T cell activation. *J Immunol* (2010) **185**:5907–14. doi:10.4049/jimmunol.1000835
35. Cubillos-Ruiz JR, Martinez D, Scarlett UK, Rutkowski MR, Nesbeth YC, Camposeco-Jacobs AL, et al. CD277 is a negative co-stimulatory molecule universally expressed by ovarian cancer microenvironmental cells. *Oncotarget* (2010) **1**:329–38.
36. Chapoval AI, Smithson G, Brunick L, Mesri M, Boldog FL, Andrew D, et al. BTNL8, a butyrophilin-like molecule that costimulates the primary immune response. *Mol Immunol* (2013) **56**:819–28. doi:10.1016/j.molimm.2013.08.003
37. Aigner J, Villatoro S, Rabionet R, Roquer J, Jimenez-Conde J, Marti E, et al. A common 56-kilobase deletion in a primate-specific segmental duplication creates a novel butyrophilin-like protein. *BMC Genet* (2013) **14**:61. doi:10.1186/1471-2156-14-61
38. Le Page C, Ouellet V, Quinn MC, Tonin PN, Provencher DM, Mes-Masson AM. BTF4/BTNA3.2 and GCS as candidate mRNA prognostic markers in epithelial ovarian cancer. *Cancer Epidemiol Biomarkers Prev* (2008) **17**:913–20. doi:10.1158/1055-9965.EPI-07-0692
39. Le Page C, Marineau A, Bonza PK, Rahimi K, Cyr L, Labouba I, et al. BTN3A2 expression in epithelial ovarian cancer is associated with higher tumor infiltrating T cells and a better prognosis. *PLoS One* (2012) **7**:e38541. doi:10.1371/journal.pone.0038541
40. Vavassori S, Kumar A, Wan GS, Ramanjaneyulu GS, Cavallari M, El Daker S, et al. Butyrophilin 3A1 binds phosphorylated antigens and stimulates human gammadelta T cells. *Nat Immunol* (2013) **14**:908–16. doi:10.1038/ni.2665
41. Sandstrom A, Peigne CM, Leger A, Crooks JE, Konczak F, Gesnel MC, et al. The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vgamma9Vdelta2 T cells. *Immunity* (2014) **40**:490–500. doi:10.1016/j.immuni.2014.03.003
42. Bas A, Swamy M, Abeler-Dorner L, Williams G, Pang DJ, Barbee SD, et al. Butyrophilin-like 1 encodes an enterocyte protein that selectively regulates functional interactions with T lymphocytes. *Proc Natl Acad Sci U S A* (2010) **108**:4376–81. doi:10.1073/pnas.1010647108
43. Valentonyte R, Hampe J, Huse K, Rosenstiel P, Albrecht M, Stenzel A, et al. Sarcoidosis is associated with a truncating splice site mutation in BTNL2. *Nat Genet* (2005) **37**:357–64. doi:10.1038/ng1519
44. Price P, Santoso L, Mastaglia F, Garlepp M, Kok CC, Allcock R, et al. Two major histocompatibility complex haplotypes influence susceptibility to sporadic inclusion body myositis: critical evaluation of an association with HLA-DR3. *Tissue Antigens* (2004) **64**:575–80. doi:10.1111/j.1399-0039.2004.00310.x
45. Mochida A, Kinouchi Y, Negoro K, Takahashi S, Takagi S, Nomura E, et al. Butyrophilin-like 2 gene is associated with ulcerative colitis in the Japanese under strong linkage disequilibrium with HLA-DRB1*1502. *Tissue Antigens* (2007) **70**:128–35. doi:10.1111/j.1399-0039.2007.00866.x
46. Pathan S, Gowdy RE, Cooney R, Beckly JB, Hancock L, Guo C, et al. Confirmation of the novel association at the BTNL2 locus with ulcerative colitis. *Tissue Antigens* (2009) **74**:322–9. doi:10.1111/j.1399-0039.2009.01314.x
47. Juyal G, Prasad P, Senapati S, Midha V, Sood A, Amre D, et al. An investigation of genome-wide studies reported susceptibility loci for ulcerative colitis shows limited replication in north Indians. *PLoS One* (2011) **6**:e16565. doi:10.1371/journal.pone.0016565
48. Moller M, Kwiatkowski R, Nebel A, Van Helden PD, Hoal EG, Schreiber S. Allelic variation in BTNL2 and susceptibility to tuberculosis in a South African population. *Microbes Infect* (2007) **9**:522–8. doi:10.1016/j.micinf.2007.01.011
49. Orozco G, Eerligh P, Sanchez E, Zhernakova S, Roep BO, Gonzalez-Gay MA, et al. Analysis of a functional BTNL2 polymorphism in type 1 diabetes, rheumatoid arthritis, and systemic lupus erythematosus. *Hum Immunol* (2005) **66**:1235–41. doi:10.1016/j.humimm.2006.02.003
50. Orlando G, Soker S, Wood K. Operational tolerance after liver transplantation. *J Hepatol* (2009) **50**:1247–57. doi:10.1016/j.jhep.2009.03.006
51. Svendsen UG, Aggestrup S, Heilmann C, Jacobsen N, Koch C, Larsen B, et al. Transplantation of a lobe of lung from mother to child following previous transplantation with maternal bone marrow. *Eur Respir J* (1995) **8**:334–7. doi:10.1183/09031936.95.08020334
52. Comerci GD Jr, Williams TM, Kellie S. Immune tolerance after total lymphoid irradiation for heart transplantation: immunosuppressant-free survival for 8 years. *J Heart Lung Transplant* (2009) **28**:743–5. doi:10.1016/j.healun.2009.04.011
53. Barth RN, Janus CA, Lillesand CA, Radke NA, Pirsch JD, Becker BN, et al. Outcomes at 3 years of a prospective pilot study of Campath-1H and sirolimus immunosuppression for renal transplantation. *Transpl Int* (2006) **19**:885–92. doi:10.1111/j.1432-2277.2006.00388.x
54. Delis S, Ciancio G, Burke GW III, Garcia-Morales R, Miller J. Donor bone marrow transplantation: chimerism and tolerance. *Transpl Immunol* (2004) **13**:105–15. doi:10.1016/j.trim.2004.05.006

55. Parulekar AD, Boomer JS, Patterson BM, Yin-Declue H, Deppong CM, Wilson BS, et al. A randomized controlled trial to evaluate inhibition of T-cell costimulation in allergen-induced airway inflammation. *Am J Respir Crit Care Med* (2013) **187**:494–501. doi:10.1164/rccm.201207-1205OC
56. Viglietta V, Bourcier K, Buckle GJ, Healy B, Weiner HL, Hafler DA, et al. CTLA4Ig treatment in patients with multiple sclerosis: an open-label, phase 1 clinical trial. *Neurology* (2008) **71**:917–24. doi:10.1212/01.wnl.0000325915.00112.61
57. Sandborn WJ, Colombel JF, Sands BE, Rutgeerts P, Targan SR, Panaccione R, et al. Abatacept for Crohn's disease and ulcerative colitis. *Gastroenterology* (2012) **143**(62–69):e64. doi:10.1053/j.gastro.2012.04.010
58. Larsen CP, Pearson TC, Adams AB, Tso P, Shirasugi N, Strobert E, et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant* (2005) **5**:443–53. doi:10.1111/j.1600-6143.2005.00749.x
59. Durrbach A, Pestana JM, Pearson T, Vincenti F, Garcia VD, Campistol J, et al. A phase III study of belatacept versus cyclosporine in kidney transplants from extended criteria donors (BENEFIT-EXT study). *Am J Transplant* (2010) **10**:547–57. doi:10.1111/j.1600-6143.2010.03016.x
60. Poirier N, Azimzadeh AM, Zhang T, Dilek N, Mary C, Nguyen B, et al. Inducing CTLA-4-dependent immune regulation by selective CD28 blockade promotes regulatory T cells in organ transplantation. *Sci Transl Med* (2010) **2**:17ra10. doi:10.1126/scitranslmed.3000116
61. Badell IR, Thompson PW, Turner AP, Russell MC, Avila JG, Cano JA, et al. Nondepleting anti-CD40-based therapy prolongs allograft survival in nonhuman primates. *Am J Transplant* (2012) **12**:126–35. doi:10.1111/j.1600-6143.2011.03736.x
62. Bekiaris V, Sedy JR, Macauley MG, Rhode-Kurnow A, Ware CF. The inhibitory receptor BTLA controls gammadelta T cell homeostasis and inflammatory responses. *Immunity* (2013) **39**:1082–94. doi:10.1016/j.immuni.2013.10.017

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Beyond CTLA-4 and PD-1, the generation Z of negative checkpoint regulators

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In the last two years, clinical trials with blocking antibodies to the negative checkpoint regulators CTLA-4 and PD-1 have rekindled the hope for cancer immunotherapy. Multiple negative checkpoint regulators protect the host against autoimmune reactions but also restrict the ability of T cells to effectively attack tumors. Releasing these brakes has emerged as an exciting strategy for cancer treatment. Conversely, these pathways can be manipulated to achieve durable tolerance for treatment of autoimmune diseases and transplantation. In the future, treatment may involve combination therapy to target multiple cell types and stages of the adaptive immune responses. In this review, we describe the current knowledge on the recently discovered negative checkpoint regulators, future targets for immunotherapy.

Keywords: cancer immunotherapy, autoimmunity, negative checkpoint regulators, TIM-3, LAG-3, TIGIT, BTLA, VISTA

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Introduction

T cells are initially stimulated through the T cell receptor (TCR) by the recognition of their cognate antigen presented by major histocompatibility complex (MHC) molecules. Optimal T cell activation requires a "second signal" provided by cosignaling molecules. Many of these molecules are members of the B7 family, and they act as rheostats that control the threshold for whether a given TCR interaction leads to activation and/or anergy. Positive costimulatory signals promote T cell proliferation and acquisition of effector function. CD28 is one such molecule that facilitates fulminant T-cell activation upon recognition of its ligands CD80 and CD86 at the surface of mature antigen-presenting cell (APC). Negative checkpoint regulators (NCRs) are molecules that down-regulate immune responses to prevent out-of-proportion immune activation, minimize collateral damage, and maintain peripheral self-tolerance.

The two NCRs that have been most actively studied are cytotoxic T lymphocyte (CTL)-associated antigen 4 (CTLA-4, CD152) and programed cell death protein 1 (PD-1, CD279) (1). They regulate immune responses at very different levels and by very different mechanisms. CTLA-4 primarily regulates the amplitude of the early stages of T cell activation by both outcompeting CD28 in binding CD80 and CD86, as well as actively delivering inhibitory signals to the T cell. PD-1 predominantly regulates effector T cell activity within tissue and tumors where the immune response is ongoing. The FDA approval of the CTLA-4 blocking antibody, Ipilimumab (Bristol-Myers Squibb) in 2011 for the treatment of advanced melanoma, followed in September and December 2014 by the approval of two PD-1 blocking antibodies, Pembrolizumab (Merck) and Nivolumab (Bristol-Myers Squibb) mark the beginning of a new era for cancer immunotherapy.

Multiple additional NCRs have been discovered in the recent years. The complex nature of the NCR pathways is only now being appreciated. These represent new promising targets for therapeutic manipulation.

TIM-3

The T-cell immunoglobulin and mucin-containing protein 3 (TIM-3) was initially identified as a specific marker of fully differentiated IFN-γ producing CD4 T helper 1 (Th1) and CD8 cytotoxic (Tc1) cells (2). TIM-3 expression is regulated by T-bet, a Th1 transcription factor (3). In addition to T cells, TIM-3 is also highly expressed on regulatory T cells (Tregs), monocytes, macrophages, and dendritic cells (DCs). TIM-3 shares a common structure with the other TIM family members consisting of an N-terminal IgV domain followed by a mucin domain, a transmembrane domain, and a cytoplasmic tail. TIM-3 does not contain any known inhibitory signaling motifs but its phosphorylation on two intracellular tyrosine residues allows for the recruitment of the Src family tyrosine kinase Fyn and the p85 phosphatidylinositol 3-kinase (PI3K) adaptor (4).

The S-type lectin Galectin-9 (Gal-9) has been identified as one of TIM-3 ligands, binding the IgV domain of TIM-3 (5). Gal-9 is a widely expressed soluble molecule upregulated by IFN-γ (6).

TIM-3 Negatively Regulates Th1 and Tc1 Responses

Binding of Gal-9 to TIM-3 causes an inhibitory signal, resulting in apoptosis of Th1 cells and cytotoxic CD8 T cells *in vitro* (5, 7).

TIM-3/Gal-9 blockade generally induces hyperproliferation of effector cells associated with increased Th1 cytokine production (5) and increased CD8 T cell cytotoxicity (8). As a consequence, blocking TIM-3-mediated signaling on T cells *in vivo* accelerates or exacerbates Th1- and Tc1-mediated diseases. Gal-9 siRNA-treated mice (5) and Gal-9 deficient hosts (9) present increased symptoms of experimental autoimmune encephalomyelitis (EAE), the mouse model of multiple sclerosis. In addition, both TIM-3 blocking antibody and TIM-3–Ig fusion protein exacerbate symptoms of EAE (5, 10, 11), type I diabetes in non-obese (NOD) mice (12), and acute graft-versus-host disease (aGVHD) (13, 14). Importantly, TIM-3 deficiency on donor T cells exacerbates EAE and aGVHD (10, 14). On the other hand, blocking this pathway can dampen allergen-induced airway inflammation by skewing the Th2 response toward a Th1 type (15).

Conversely, activating the TIM-3 pathway ameliorates various disease models. Gal-9 overexpressing mice are protected from aGVHD (14). Recombinant Gal-9 administration suppresses EAE (5, 9) and prolongs the survival of fully allogeneic skin or cardiac transplants (16–18). Gal-9 expressing islets are also protected from rejection by NOD T cells (19). In all these models, the protection conferred by Gal-9 is associated with a decrease in IFN-γ producing Th1 and/or Tc1 cells.

Taken together, these data strongly support the hypothesis that the upregulation of TIM-3 on activated T cells and its interaction with Gal-9 plays a critical role in attenuating and/or terminating both CD4 Th1 and CD8 Tc1 immune responses.

TIM-3 Regulates Th17/Tregs Differentiation

Whether and how TIM-3 and Gal-9 regulate Th17 cells is unresolved. While some studies show a negative effect of Gal-9 on both Th1 and Th17 development *in vivo* (16, 20), some studies show an impact on Th1 only (19). Gal-9 potentiates Treg conversion, and suppresses differentiation of Th17 cells *in vitro* (20, 21). As a result, Gal-9 administration ameliorates collagen-induced arthritis (CIA) by decreasing the levels of IFN-γ and IL-17 in the joints (20). However, one study demonstrated that Gal-9 suppression of Th17 development is TIM-3-independent (9).

In vitro, blocking TIM-3 promotes both Th1 and Th17 cytokine production by human and mouse CD4 T cells (8, 22). Similarly, *in vivo* TIM-3 blockade increases both Th1 and Th17 cells (8). However, TIM-3 blockade does not increase incidence and severity of Th17-mediated EAE but alters the pattern of inflammation due to differential effects on Th1 versus Th17 cells (10). TIM-3 blockade also inhibits Treg differentiation *in vitro* (8) and *in vivo* (12). As a result, TIM-3 deficient mice cannot be tolerized by high-dose aqueous antigen administration (11) and TIM-3 blockade abrogates Treg-mediated tolerance to allogeneic islets induced by donor-specific transfusion and costimulatory blockade (12).

Overall, evidence suggests that TIM-3 and Gal-9, possibly independently of each other, are involved in the differential regulation of Tregs and Th17 differentiation and contribute to T cell tolerance. One mechanism proposed is that TIM-3 negatively regulates IL-6 production by CD4 T cells. Therefore, blocking TIM-3 induces IL-6 production, which then antagonizes Treg differentiation and promotes IL-17 production by naive CD4 T cells (8).

TIM-3 Regulates Innate Cell Activation/Expansion

TIM-3 is highly expressed by innate immune cells including monocytes, macrophages, and DCs, and regulates their function in several ways.

In some circumstances, TIM-3 acts as a negative regulator of myeloid cell activation. Monney et al. first showed that a blocking TIM-3 antibody induces increased activation of macrophages (2). In addition, TIM-3 blockade during the innate immune phase of the response to coxsackievirus B3 (CVB3) infection exacerbates inflammatory heart diseases (23).

TIM-3 expression on macrophages can dampen TLR4-mediated inflammatory reactions and damage (24). Moreover, expression of TIM-3 and TLR4 is reciprocally regulated (25, 26). TIM-3 blockade enhances macrophage responsiveness to LPS stimulation, exacerbates sepsis (24), and enhances ischemia reperfusion injury damage in mouse liver transplantation (27). In these cases, the effect of TIM-3 blockade is dependent on intact TLR4 expression. TIM-3 overexpression on macrophages as observed in chronic hepatitis C virus (HCV) infection, or by transgenic overexpression, is associated with diminished cytokine production upon stimulation (24, 26). However, TIM-3 overexpressing macrophages in hepatocellular carcinoma patients promote tumor cell growth via IL-6 production (28).

On the other hand, several studies have indicated that TIM-3 can promote activation and inflammatory cytokine production by innate immune cells. Triggering the TIM-3 pathway on DCs and monocytes via Gal-9 treatment or agonistic anti-TIM-3 antibody synergizes with TLR ligands to promote their activation (29). Gal-9 alone promotes the secretion of proinflammatory cytokines by TIM-3 expressing human and mouse monocytes and DC (30). In addition, Gal-9 treatment reverses immune suppression in tumor-bearing hosts and enhances survival by promoting the maturation of TIM-3 expressing DCs thus promoting adaptive immunity (31). These apparently contradictory results might be explained by a differential effect of TIM-3 on macrophages versus DCs. Other binding partners for TIM-3 and/or Gal-9 can also mediate this differential effect. In addition, the fact that TIM-3 and Gal-9 can act as both receptors and ligands with regard to signaling has to be taken into account.

Several reports have indeed established that there is a reciprocal signal transmitted to the Gal-9 expressing innate immune cell. Therefore, a TIM-3–Ig fusion protein often used as a blocking reagent may trigger a signal to Gal-9 expressing cells independently of its blocking of TIM-3. For example, the interaction of Gal-9 expressed on macrophages with TIM-3 expressed on Th1 enhances their bactericidal activity. This effect can be mimicked on macrophages by treatment with TIM-3–Ig (32, 33). Importantly, this effect is lost in Gal-9-deficient macrophages (32). Kuchroo et al. created TIM-3 Tg mice where TIM-3 overexpression is controlled by the human CD2 promoter and restricted to T cells. These mice display dampened T cell immunity resulting in increased tumor progression that is linked to the expansion of granulocytic myeloid-derived suppressor cells (MDSCs). Thus, TIM-3 expressed on T cells is sufficient to trigger a signal via Gal-9 in MDSCs and promote their expansion (34). In conclusion, both TIM-3 and Gal-9 are expressed and can signal in innate immune cells. Thus, cis and trans interactions might occur and the final effect results from the integration of all of these signals (35).

Additional Ligands and Partners for TIM-3 and Gal-9

As mentioned above, TIM-3 and Gal-9 may each have multiple binding partners. Several studies have found that Gal-9 may act through counter structures that are not TIM-3. First, Gal-9-mediated cell death of Th1 cells is not completely abolished in TIM-3-deficient cells (5). In addition, Gal-9 induces proinflammatory cytokine production by T helper cells, suppresses Th17 development, and induces plasma cell apoptosis in a TIM-3-independent manner (9, 36, 37). A recent study also reported that TIM-3 does not act as a binding partner for Gal-9 on human T cells (38).

Early crystal structure studies of TIM-3 have revealed a Gal-9 independent ligand-binding surface in the IgV domain (39) and several TIM-3 additional binding partners have since been uncovered. Like other members of the TIM family, TIM-3 also binds phosphatidylserine (PtdSer), exposed at the surface of apoptotic cells (40). Binding to PtdSer by TIM-3 mediates the uptake of apoptotic cells by TIM-3 expressing phagocytes such as CD8α DC and appears crucial for the clearance of apoptotic cells *in vivo* (41). TIM-3 blockade thus prevents uterine macrophages from clearing apoptotic cells and the resulting local inflammation increases fetal resorption (42).

TIM-3 also interacts with the high-mobility group protein B1 (HMGB1). This interaction prevents the trafficking of nucleic acids into endosomes and decreases stimulation of endosomal TLR pathways thereby preventing tumor recognition by TIM-3 expressing DCs and promoting tumor escape (43).

Finally, TIM-3 has recently been described to interact with the carcinoembryonic antigen cell adhesion molecule 1 (CEACAM-1) in both cis and trans through their N-terminal domains. CEACAM-1 endows TIM-3-mediated inhibitory function by facilitating TIM-3 surface expression (44). In conclusion, TIM-3 and Gal-9 can both use multiple binding partners mediating various outcomes in both T cells and innate immune cells unraveling a very complex functional role.

TIM-3 Regulates T Cell Exhaustion

TIM-3 expression has been described to mark the most dysfunctional CD8 T cells in various chronic viral infections in both human and mice such as human immunodeficiency virus (HIV), HCV, and lymphocytic choriomeningitis virus (LCMV) (45–47). This state of T cell dysfunction, called T cell exhaustion, caused by chronic antigenic stimulation is characterized by the failure to respond further, proliferate, and exert effector functions such as cytotoxicity and cytokine secretion in response to antigen stimulation. These cells as discussed thereafter, often co-express other inhibitory molecules.

Similarly, TIM-3 and PD-1 are co-expressed on most CD4 and CD8 T cells infiltrating solid tumors or in hematologic malignancy in mice and these cells are dysfunctional (48, 49). TIM-3 and PD-1 expression is also upregulated on exhausted tumor-specific CD8 T cells in the blood of melanoma and lymphoma patients (50, 51). In both chronic viral infection and cancer, blocking TIM-3 *ex vivo* or *in vivo* increases the functionality of exhausted T cells and synergizes with PD-1 blockade to restore viral control (46, 52) or to inhibit tumor growth (48, 49).

TIM-3 expression on tumor-infiltrating lymphocytes (TILs) also defines highly suppressive Tregs in both human and mouse tumors (53–55). As a result, TIM-3 blockade and Treg depletion have a synergistic effect on tumor growth inhibition (54).

LAG-3

The lymphocyte-activated gene-3 (LAG-3, CD223) is a surface molecule highly homologous to CD4 in structure, but with less than 20% identity at the amino acid level (56). Like CD4, LAG-3 binds to MHC class II molecules, but with a much higher affinity (57).

LAG-3 is expressed on activated CD4 and CD8 T cells, and on activated Tregs (58) and Tr1 cells (59, 60). It is also expressed on a subset of NK cells (61) B cells (62) and plasmacytoid DCs (63). In resting T cells, LAG-3 is localized and degraded within the lysosomal compartments (64, 65). After stimulation, LAG-3 is rapidly translocated to the cell surface where its expression is regulated by two TCR-induced metalloproteases, ADAM10 and ADAM17. LAG-3 cleavage from the cell surface by these

metalloproteases allows for normal T-cell activation (66). As a result, LAG-3 is only transiently expressed at the surface of activated T cells stimulated in acute conditions, although it remains high on T cells stimulated within tolerizing environments (58, 67).

LAG-3 associates with the TCR:CD3 complex following TCR engagement and negatively regulates signal transduction (68). A single lysine residue (K468) within a conserved "KIEELE" motif in the cytoplasmic tail of LAG-3 is essential for interaction with downstream signaling molecules and inhibitory function (69).

LAG-3 Prevents Autoimmunity in Mice

LAG-3 deficiency alone does not induce autoimmunity in non-autoimmune-prone mouse strains (70–72) and does not induce major alterations in T cell development or function but a reduced NK cell cytotoxicity (70). Probably for that reason, LAG-3-deficient mice present a reduced ability to control tumor growth (72). However, LAG-3 blockade or LAG-3 deficiency accelerates diabetes in the predisposed NOD mice (71, 73). In addition, combined LAG-3 and PD-1 deficiency induces massive autoimmune conditions and early death in several different genetic backgrounds (71, 72). These clearly identify LAG-3 as a non-redundant negative T cell regulator.

LAG-3 Negatively Regulates T Cell Activation

Multiple evidences suggest that LAG-3 signaling directly inhibits primary activation of T cells *in vitro* and *in vivo*.

LAG-3 blockade induces increased proliferation and cytokine production by T cells activated *in vitro* (74, 75). *In vivo*, LAG-3-deficient T cells exhibit a delay in cell cycle arrest resulting in a larger memory T cell pool following simian virus (SV) infection (76). LAG-3-deficient donor T cells also induce more severe aGVHD due to increased proliferation and enhanced effector functions (77).

LAG-3 also plays both a direct role and an indirect role in maintaining the tolerogenic state of CD8 T cells *in vivo*. LAG-3 deficiency on CD8 T cells prevents the development of transgenic CD8 T cell tolerance, these cells are exposed to cognate self-Ag (78). In a different model of CD8 T cell tolerance induced by allogeneic bone marrow transplantation and costimulation blockade, LAG-3 blockade also abrogates donor-specific CD8 T cell tolerance (79). However, in this system, LAG-3 is not intrinsically required on CD8 but must be expressed by other cells.

LAG-3 Regulates the Induction and Suppressive Ability of Tregs and Tr1 Cells

LAG-3 is a marker of IL-10 producing Tr1 cells in both mice and humans (59, 60). Importantly, LAG-3 is one of the most overexpressed genes on CD4 transgenic T cells stimulated within a tolerizing environment (58, 78).

LAG-3 likely plays a crucial role in Tr1 induction and its function as ectopic LAG-3 expression in CD4 T cells confers a suppressive activity and blocking LAG-3 inhibits the suppressive function of Tr1 cells *in vitro* and *in vivo* (58). LAG-3 crosslinking on human T cells also induces a functional unresponsiveness that can be reversed by IL-2, consistent with a Tr1 phenotype (80).

LAG-3 is also highly expressed by activated natural Tregs (58). LAG-3 plays a role in modulating Treg induction/expansion as LAG-3 deficiency on T cell or LAG-3 blockade prevents Treg conversion in favor of a TH1 skewing (81). The importance of LAG-3 for Treg-mediated suppression is controversial. In one study, LAG-3-deficient Tregs cannot suppress homeostatic proliferation (82), whereas two other studies showed no difference in LAG-3-deficient or -sufficient Tregs to suppress homeostatic proliferation and aGVHD (77, 81). It is possible that LAG-3 is necessary for Treg-mediated suppression at high Effector/Treg ratios while being dispensable at lower ratios.

In these last two studies, LAG-3 expression on conventional T cells however, regulated their susceptibility to Treg-mediated suppression. LAG-3-deficient T cells undergo increased homeostatic expansion when transferred in a lymphopenic host (82) and LAG-3 blockade also increases homeostatic expansion but only if Tregs are present (81). This involves a novel Treg-mediated suppression mechanism following MHC class II acquisition by Tregs through trogocytosis and subsequent inhibition of LAG-3 expressing conventional T cells (77). Thus, LAG-3 appears as a crucial molecule involved in both the development and function of suppressive T cells.

LAG-3 Regulates Innate Cell Activation

Similar to other NCRs, LAG-3 is bidirectional in its signaling capacity and modulates DC activation by inducing downstream signaling via MHC class II molecules. LAG-3 expressed on activated T cells induces DC maturation with the production of TNFα and IL-12 *in vitro*. As a result, LAG-3 blockade in DC:T cell cocultures, prevents DC activation, and inhibits rather than increases T cell proliferation (83). This T-cell-mediated effect on DC can be mimicked by soluble LAG-3-Ig fusion protein (84). As a result, LAG-3–Ig acts as an adjuvant increasing Th1 and cytotoxic T cell responses to soluble antigen *in vivo* (85). Similarly, LAG-3–Ig administered together with irradiated tumor cells induces tumor regression and increases tumor cell-specific CD8 T cell responses (86). However, during Treg:DC interactions, LAG-3 engagement with MHC class II inhibits DC activation. In this case again, LAG-3-mediated signaling is not required but its binding to MHC II molecules initiates an inhibitory signaling pathway that suppress DC maturation (87). MHC II engagement through LAG-3 or crosslinking induces several pathways that have to be finely regulated to lead to cell activation or inhibition (88). Additional signals differentially expressed by activated T cells and Tregs such as CD40L might also influence the outcome of this interaction (83).

Additional LAG-3 Ligands

As for other NCRs, two other binding partners for LAG-3 have been described which are expressed in the tumor microenvironment: the Liver sinusoidal endothelial cell lectin (LSECtin) and Galectin-3 (Gal-3). Engagement of LAG-3 by LSECtin expressed in melanoma cells inhibits IFNγ production by effector T cells and increases IL-10 production by Tregs (89). Gal-3, a galactoside-binding soluble lectin is expressed in several cell types and involved in a broad range of physiological

and pathological processes. Gal-3 binds to LAG-3, and LAG-3 expression is necessary for Gal-3-mediated suppression of tumor-specific CD8 T cells. Gal-3 deficiency on both T cells and the host improves tumor-specific CD8 T cell response suggesting both cis and trans interactions between the two molecules (90).

LAG-3 Regulates T Cell Exhaustion in Cancer and Chronic Infections

In addition to PD-1 and TIM-3, LAG-3 is also upregulated and maintained in exhausted T cells in both chronic viral infections and cancer. LAG-3 is upregulated on virus-specific CD8 T cells in chronic LCMV infection. A functional role of LAG-3 in exhaustion is suggested by the fact that LAG-3 blockade synergized with PD-1 blockade to reverse exhaustion and improve viral control (91). LAG-3 expression on HIV-specific CD4 and CD8 T cells is also correlated with disease progression. Interestingly in HIV patients, LAG-3 and PD-1 are expressed on distinct subsets of exhausted T cells (92). LAG-3 and PD-1 are co-expressed on TILs in ovarian and on tumor-specific CD8 T cells in the blood of ovarian cancer patients (93). LAG-3 and PD-1 are also co-expressed on CD4 and CD8 TILs in various mouse tumor models (72). LAG-3 blockade alone does not always reverse the exhausted phenotype but can synergize with PD-1 blockade to improve effector functions and control viral load (91, 92) or induce tumor regression (72, 93).

TIGIT

The T cell immunoreceptor with Ig and ITIM domains (TIGIT/Vstm3/WUCAM/VSIG9) is a novel member of the immunoglobulin super family (IgSF). TIGIT was recently identified by two independent groups through a genomic search for genes specifically expressed in T cells and bearing a structure similar to other immunomodulatory receptors (94, 95). TIGIT is a type 1 transmembrane protein containing an IgV extracellular domain and an immunoglobulin tail tyrosine (ITT)-like phosphorylation motif followed by an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic tail.

TIGIT pairs with CD226/DNAM-1 (DNAX Accessory Molecule-1) to form an emerging pathway that has striking similarities to the CTLA-4/CD28 pathway. CD226 and TIGIT bind the same set of ligands, the two nectin-family members poliovirus receptor (PVR) (CD155/Necl-5/Tage4) and poliovirus receptor-related 2 PVRL2 (CD112), and compete with each other (96). Both ligands are members of the nectin-like family, are widely expressed outside the hematopoietic system on fibroblasts and endothelial cells, and are involved in cell adhesion and motility. Notably, PVR is overexpressed in several tumor cells types (97, 98) and can be induced by Ras activation and genotoxic stress (99, 100). PVR is also induced by TLR ligand-activated APCs (101). Whereas CD226 is widely expressed on most immune cells (102), TIGIT is absent on naive T cells, but is expressed on activated and memory T cells, Tregs (94) and on NK cells and NKT cells (95) in mice and humans. Human TIGIT engagement by PVR induces a tyrosine phosphorylation on the ITT domain. This results in the recruitment of the phosphatase SHIP1 through different cytosolic adaptors leading to inhibition of phosphatidylinositol 3-kinase (PI3K), MAPK, and NF-kB signaling (103, 104). In mice, phosphorylation of either the ITT or the ITIM domain is sufficient for TIGIT-mediated inhibition (105).

CD226 Costimulates NK and T Cell Responses

PVR recognition by CD226 potentiates CD8 T cell and NK cell cytotoxicity toward tumor cells (97, 106, 107). Notably, CD226 is a crucial costimulatory molecule for CD8 T cells when activated by non-professional APC such as B cells but is dispensable when T cells are activated by professional APCs (108). As a result, CD226-deficient mice have impaired anti-tumor and antiviral T cell responses (109, 110). Upon engagement, CD226 is phosphorylated and interacts with LFA-1 inducing their recruitment to lipid rafts (111–114). CD226 deficiency thus impairs immunological synapse formation between CD8 T cells and target cells preventing the deliverance of the cytotoxic payload necessary for target cell killing (115). CD226 also regulates CD4 T cell expansion and cytokine production. CD226 blockade decreases Th1 differentiation and suppresses EAE while PVR deficiency decreases Th2 polarization (101, 102).

The polymorphism variant Gly306Ser of CD226 has been associated with susceptibility to multiple autoimmune diseases such as SLE, autoimmune thyroid disease, Type 1 diabetes, MS, and Celiac disease (116, 117).

TIGIT Has T-Cell-Intrinsic Inhibitory Function

Several reports attest that TIGIT negatively regulates T cell activation. While TIGIT deficiency alone does not induce overt autoimmunity, TIGIT pathway blockade exacerbates several immune diseases. TIGIT-deficient mice are more susceptible to EAE (118) and blocking TIGIT results in more rapid CIA and EAE diseases onset (96). TIGIT deficiency also induces neurological dysfunction in EAE susceptible, myelin oligodendrocyte glycoprotein (MOG)-specific TCR transgenic 2D2 mice (118). Finally, TIGIT-deficient T cells induce more severe GVHD (96).

Conversely, increased TIGIT function on T cells has been shown to ameliorate a variety of autoimmune disease models in mice. Soluble TIGIT decreases CIA and mice overexpressing TIGIT in T and B cells are protected against EAE (96). Lentiviral overexpression of TIGIT in CD4 T cells decreases their ability to mediate bone marrow damage and lengthens survival time in a mouse model of aplastic anemia (119).

The mechanism of TIGIT-mediated T cell inhibition is unclear. Some evidences suggest that TIGIT can directly inhibit T cell activation. *In vitro*, TIGIT engagement by an agonistic antibody decreases both human and mouse T cell activation when stimulated with anti-CD3 and anti-CD28 (119, 120). Conversely, TIGIT knockdown increases T cell proliferation and effector cytokine production while decreasing IL-10 production (119). This suggests that the negative downstream signaling via TIGIT could arrest T cell activation. However, TIGIT can also indirectly inhibit T cell activation by opposing the CD226-mediated positive costimulatory signal either through ligand competition or CD226 inhibition. As evidence, when T cells are activated with PVR transfected artificial APCs, a blocking TIGIT antibody increases T cell proliferation while soluble TIGIT-Ig decreases

it (96). TIGIT deficiency or blockade also increases T cell proliferation to anti-CD3 and PVR-Ig stimulation. Importantly, when T cells are activated in the presence of PVR, TIGIT-mediated inhibition seems dependent on CD226, as CD226 blockade annihilates the positive impact of TIGIT blockade (121). FRET studies indicate that TIGIT and CD226 directly interact at the cell surface and that this interaction impairs CD226 homodimerization and function (121).

TIGIT Regulates DC Immunostimulatory Function

As mentioned before, most immunoregulatory molecules function in a bidirectional way and TIGIT also induces a reciprocal signal in PVR expressing APC. PVR engagement on DC with TIGIT-Ig induces IL-10 while suppressing pro-inflammatory cytokines production such as IL-12. Upon TIGIT ligation, PVR is phosphorylated and elicits downstream signaling in DCs, which then inhibits T cell responses by producing IL-10. In addition, TIGIT blockade exacerbates T cell responses only in the presence of DC. Finally, TIGIT–Ig-mediated inhibition of delayed type hypersensitivity (DTH) reactions *in vivo* is dependent of IL-10 (94). All these suggest that TIGIT negative regulation of T cell is indirect and mediated at least in part through modification of the immunostimulatory function of APCs.

In addition, TIGIT also exerts a direct inhibitory role on APCs as TIGIT-deficient APCs are better at promoting T cell proliferation. The maximal APC-induced T cell proliferation is achieved by combining both deficient T cells and deficient APCs suggesting that TIGIT has synergistic roles on T cells and APCs (118).

TIGIT Negatively Regulates NK Cell Effector Function

In addition to T cell inhibition, TIGIT negatively regulates NK cell cytotoxicity and cytokine production. TIGIT blockade increases NK cell cytotoxicity toward PVR expressing targets. Interestingly, TIGIT inhibition is dominant over the coactivation mediated by CD226 on NK cells (95, 105) whereas the net effect of PVR-T-cell interaction appears costimulatory (96). This might be due to additional inhibitory mechanisms preventing NK cytotoxicity. Indeed, NK cells also express CD96, another inhibitory receptor that also competes with CD226 for PVR binding (122). In addition, only co-blockade of TIGIT and MHC class I increases NK-mediated cytotoxicity against fibroblasts. TIGIT may thus represent an "alternative self" mechanism for MHC class I inhibition, preventing damage to self-tissue (95). Using mutants of TIGIT transfected into a YTS NK cell line and PVR transfected 7721.221 target cells, Fan et al. deciphered the mechanisms involved in TIGIT-mediated inhibition of NK cells. In these cells, direct TIGIT-mediated negative downstream signaling results in impaired granule polarization (104) and IFN-γ production (103).

TIGIT Promotes Treg Differentiation and Defines Activated, Highly Suppressive Tregs

TIGIT transcription is directly regulated by FoxP3 (123). TIGIT promotes inducible Treg differentiation as TIGIT deficiency decreases Treg conversion *in vitro*. Conversely, T cells overexpressing TIGIT generate greater frequencies of Tregs (124). *In vivo*, TIGIT expression defines a subset of activated natural Tregs with superior suppressive capacity in both humans and mice (125). TIGIT-expressing Tregs express higher amount of PD-1, CTLA-4, LAG-3, and TIM-3 and produce more IL-10 and Fibrinogen-like protein 2 (Fgl2). Notably, Fgl2 production is induced by TIGIT engagement and is responsible for the increased suppressive ability of TIGIT Tregs. These Tregs specifically suppress Th1 and Th17 responses *in vivo*, while promoting Th2 responses in an Fgl2-dependent manner (124). In human, co-expression of TIGIT and Fc receptor-like protein 3 (FCRL3) identifies Helios + memory Tregs (125, 126). Thus, as other NCRs, TIGIT is critically involved in Treg development and suppressive function.

TIGIT Regulates T Cell Exhaustion

Similar to PD-1, TIM-3, and LAG-3, TIGIT is upregulated on exhausted T cells in both chronic viral infections and cancer (121). A gene signature-based approach identified TIGIT expression as a marker for tumor-associated T cells. The TIGIT:CD3 ratio is increased on T cells in multiple human tumors compared to the corresponding normal tissues indicating that TIGIT is specifically upregulated in tumor-infiltrating T cells. Indeed, TIGIT is highly expressed on CD8 T cells co-expressing PD-1 infiltrating non-small cell lung carcinoma (NSCLC) and colorectal carcinoma (CRC) as well as several mouse tumor models. TIGIT is also elevated on CD4 and CD8 T cells in the blood of cancer patients. Whereas PD-L1 or TIGIT blockade alone have little effect, PD-L1 and TIGIT co-blockade dramatically improves CD8-mediated control of tumor growth leading to complete rejection in the majority of mice. Importantly only PD-L1 and TIGIT co-blockade elicit IFNγ and TNFα production by CD8 TILs (121).

In mice chronically infected with the Clone 13 strain of LCMV, TIGIT is highly expressed by PD-1^{high} exhausted T cells and TIGIT blockade acts synergically with PD-1 blockade to improve CD8 T cell effector function and viral control. TIGIT conditional knock down in T cells also improves antiviral T cell response and viral control. However, as mentioned above, TIGIT inhibition of T cells seems mediated by CD226 disruption as CD226 blockade annihilates the curative impact of TIGIT and PD-L1 co-blockade on both tumor growth and chronic infection (121). Interestingly, CD226 expression is downregulated on virus-specific CD8 T cells in both human HIV and mouse LCMV chronic infection reinforcing the proposition that the costimulatory pathway mediated by CD226 is disrupted in chronically exhausted T cells (127).

BTLA

B and T lymphocyte attenuator (BTLA) was identified as another NCR in the IgSF structurally related to CTLA-4 and PD-1 (128, 129). BTLA expression is limited to lymphoid tissues, with highest expression on B cells, but also significant on both αβ and γδ T cells, mature DCs, and macrophages (128, 129). BTLA is expressed on naïve T cells, transiently upregulated upon TCR engagement, but is down regulated on fully activated T cells. However, in similarity with LAG-3, BTLA remains high on T cells rendered anergic *in vivo* (130). Its absence on fully activated T cells distinguishes it from other B7 family members

like PD-1 and CTLA-4 (130, 131). Unique for a IgSF member protein, is the counter-receptor that BTLA binds. BTLA binds to the herpesvirus entry mediator (HVEM), which is a member of the tumor necrosis factor receptor superfamily (TNFRSF) (132, 133). HVEM is expressed on T cells, B cells, NK cells, DCs, and myeloid cells (133). HVEM was originally described as a receptor for HSV glycoprotein D, but interacts with multiple other ligands in addition to BTLA, including CD160 and LIGHT. CD160 is another negative receptor on T cells (134), while LIGHT is a costimulatory molecule. Crosstalk across protein families and the use of multiple binding partners by each protein are indicative of the widespread complexity of the system of negative checkpoint regulation. Like PD-1 and CTLA-4, BTLA contains two conserved ITIMs in its cytoplasmic tail (129). BTLA engagement is associated with phosphorylation at those two ITIMs motifs resulting in the association of the Src homology domain 2 (SH2)-containing protein tyrosine phosphatases SHP-1 and SHP-2 (128, 129, 135) with either the TCR or the BCR.

BTLA Negatively Regulates T Cell Activation

In vitro studies demonstrate a direct negative activity of BTLA on T cell proliferation and cytokine production. BTLA-deficient T and B cells show enhanced proliferation in response to anti-CD3 and anti-IgM respectively (128, 129). Retroviral overexpression of BTLA in DO11.10 cells or agonist BTLA antibody suppresses anti-CD3-induced IL-2 production (129, 135). In addition, HVEM–Fc fusion protein inhibits T cell proliferation in response to multiple agonists (132). Interestingly, BTLA signaling induced by agonist antibody can act on T cell proliferation as far as 16 hours after TCR engagement, suggesting that the critical point of impact is during the later, more stable T cell–APC interactions (135).

BTLA Regulates Peripheral Tolerance

BTLA deficient mice gradually develop elevated anti-self antibodies, an increased number of activated CD4 T cells in the periphery and inflammatory cell infiltration of multiple organs. This can progress to development of a hepatitis-like disease and overall reduced survival (136). Consistent with this breakdown in peripheral tolerance, BTLA-deficient mice are resistant to the induction of T cell tolerance to an oral antigen or to high-dose antigen administration (137). In addition, BTLA-deficient ovalbumin-specific OT-I CD8 T cells cannot be tolerized by encounter with their cognate antigen *in vivo* and cause diabetes in RIP-mOVA recipient mice (137). BTLA-deficient mice also present increased susceptibility to EAE (129) and mice deficient in either BTLA or HVEM present heightened T cell and NKT cell responses to Con A and exhibit increased morbidity and mortality to Con A-mediated T cell-dependent autoimmune hepatitis (138, 139). Conversely, BTLA engagement leads to the induction of tolerance. An agonistic BTLA antibody prolongs heart allograft survival by suppressing alloreactive T cell responses and inducing IL-10-producing Tregs (140). In addition, a single dose of agonist BTLA antibody prevents the development of GVHD if given at the time of transplantation (141). In these models, BTLA engagement seems to favor the expansion of Tregs over T effector cells, adjusting the balance toward tolerance.

BTLA Regulates Innate Immunity

More recent studies have shown that BTLA is also able to regulate multiple lineages of cells within the innate immune system. BTLA-deficient mice on the RAG-deficient background are less susceptible to *Listeria Monocytogenes* infection (142). The authors reported that the loss of BTLA in the CD8α DC compartment prevents Listeria expansion within these cells. It is known that CD8α + DCs are necessary for Listeria expansion and dissemination within the host. In wild-type mice, BTLA normally suppresses Fas/FasL signaling in DCs to allow high levels of Listeria to grow and thereby induce potent protective CD4 and CD8 responses. In BTLA-deficient DCs, where Fas/FasL is enhanced, Listeria burden is reduced as is the adaptive immune response keeping it in check. Therefore, BTLA can exert direct immunoregulatory effects within the DC compartment.

Possessing both a rearranged specific TCR and rapid innate functions, γδ T cells are poised between the innate and adaptive arms of the immune response. BTLA appears to play a major role in both their homeostasis and function. The pool of γδ T cells is maintained through competition for survival signals from IL-7 or IL-15. In γδ T cells, IL-7 increases BTLA levels on the cell surface, which in turn, restricts their expansion and negatively regulates IL-17 and TNFα. BTLA-deficient mice thus exhibit enhanced disease in a γδ T cell-dependent model of dermatitis whereas an agonistic BTLA antibody reduces inflammation (143).

A particular role for BTLA has been described for Vγ9Vδ2 T cells. This subset is thought to have functions in tumor immune surveillance. In human Vγ9Vδ2 T cells, BTLA interaction with HVEM–Fc fusion protein negatively regulates Vγ9Vδ2 T-cell proliferation to both TCR-dependent and -independent activation. During TCR-mediated activation, BTLA clusters to the TCR and decreases phosphorylation of ZAP-70 and Erk1/2. BTLA blockade increases TCR signaling and restores the ability of human Vγ9Vδ2 T cells to react to HVEM expressing lymphoma cells (144). Although these suppressive activities show similarity to PD-1 and CTLA-4, the primarily naïve and central memory expression of BTLA distinguishes it from these immunoregulators, and gives BTLA its own niche.

In a transfer model of colitis, HVEM expressed on a radioresistant cell population interacting with BTLA was found to be critical in preventing inflammation (145). Surprisingly, BTLA expression on the donor T cells had a minor impact, and BTLA expression was more important in the recipient RAG-deficient mice. These data suggest that these molecules should be examined outside of the usual T cell–APC focus.

BTLA as a Ligand Demonstrates T Cell Pro-Survival Function

A wealth of reports demonstrates a negative receptor function for BTLA. However, like many of the immunoregulatory molecules that can act as both receptors and ligands, BTLA also appears to induce a reciprocal positive pro-survival signal in HVEM-expressing T cells. Indeed, BTLA has been implicated in sustaining the survival of HVEM-expressing effector and memory T cells in various mouse models (145–147). Particularly, during vaccinia virus infection, HVEM expression on T cells and BTLA expression on the host are both necessary for the development of

a protective response. In this model, BTLA expressed by APCs functions as a ligand that delivers positive signals in trans to HVEM expressing T cells (147).

An important point is that T cells express both HVEM and BTLA and several reports also demonstrate that BTLA expression by T cells is also critical for their survival. In a GVHD model, transfer of BTLA-deficient donor cells results in an impaired anti-host response due to a loss in donor T cell survival (148). Similarly, BTLA-deficient T cells do not induce increased colitis when transferred into RAG-deficient mice because of an impaired accumulation (145). A positive correlation has also been described between BTLA expression and the ability of human T cells to generate recall responses to the Mycobacterium tuberculosis antigen Ag85B (149). Sakoda et al. confirmed these findings and showed that expression of the extracellular domain of BTLA is sufficient to restore the survival of BTLA-deficient T cells during GVHD, further demonstrating the role of BTLA on T cells as a ligand in this model (150). BTLA-deficient T cell survival is also rescued with BTLA–Fc fusion protein (150, 151). Cellular BTLA and BTLA–Fc induce the recruitment of TNF receptor-associated factor 2 (TRAF2) to HVEM, promoting NF-κB activation and cell survival (151). Importantly, BTLA function as a ligand to induce pro-survival signal in GVHD is associated with its expression on donor T cells but not on recipient cells (150). A BTLA–HVEM interaction in cis on T cell could explain these findings. However, one report demonstrates that HVEM–BTLA cis interaction competitively inhibits HVEM activation by ligands expressed in the surrounding microenvironment rather suggesting a bystander T:T trans interaction involved in the BTLA–HVEM-mediated survival effect (152).

BTLA Negatively Regulates Tumor Immunity

HVEM is expressed in 26 of 40 melanoma cell lines and moderately to strongly expressed on 75% of human melanoma metastases (153). BTLA and PD-1 are co-expressed on tumor-specific CD8 T cells in melanoma patients (153, 154) and these cells are dysfunctional (154). However, contrary to PD-1, BTLA upregulation seems to occur independently of the functional exhaustion driven by high antigen load (154). BTLA can be downregulated by vaccination with peptide and CpG oligodeoxynucleotides with a resulting loss of sensitivity to HVEM-mediated suppression of cytokine production (153). Importantly, BTLA blockade synergizes with PD-1 and TIM-3 blockade in enhancing proliferation and cytokine production by tumor-specific T cells *in vitro* indicating a non-redundant role for BTLA (154). Similarly, BTLA blockade combined with active immunization enhanced anti-tumor immunity (155) and can lead to regression of large adenocarcinomas in mice (156). However, as discussed above, the effect of BTLA may not be completely negative. BTLA expressing TILs appear more proliferative to IL-2. This may be because BTLA expression is higher on newer central memory type cells that are less likely to be exhausted. In addition, BTLA positive cells present reduced sensitivity to activation-induced cell death. These data indicate that BTLA may extend the life of TILs, but also maintain quiescence (157).

VISTA

The V-domain Ig suppressor of T cell activation (VISTA) also known as PD-1 homolog (PD-1H) has recently been identified by our group and others as a novel NCR in the IgSF. VISTA is a type I transmembrane protein with a single IgV domain with sequence homology to the IgV domains of the members of CD28 and B7 families (158, 159). VISTA cytoplasmic tail domain contains two potential protein kinase C binding sites as well as proline residues that could function as docking sites, suggesting that VISTA could potentially function as both a receptor and a ligand. VISTA does not contain ITIM-like motifs. Modeling algorithms have suggested homology to either PD-1 (158) or PD-L1 (159). The counter structures interacting with VISTA have not been identified yet.

Unlike PD-L1, VISTA expression is restricted to the hematopoietic compartment. It is constitutively and highly expressed on CD11b myeloid cells such as neutrophils, monocytes, macrophages, and DCs, and expressed at lower levels on naïve CD4 and CD8 T cells and Tregs both in humans (160) and mice (158, 159).

VISTA Regulates Peripheral Tolerance

The negative regulatory function of VISTA is clearly demonstrated in deficient mice. VISTA-deficient mice demonstrate an age-related proinflammatory signature, with elevated serum cytokines, spontaneous T cell activation, and chronic multi-organ inflammation. Nonetheless, single VISTA deficiency as well as VISTA/PD-1 and VISTA/PD-L1 double deficiencies do not induce overt autoimmunity in the absence of other predisposing factors (161–163).

However, VISTA deficiency on the 2D2 transgenic EAE susceptible background dramatically increases disease incidence and severity with 60% of mice dying by 2–3 months of age (163). Combined deficiency of VISTA and PD-1 further increases disease penetrance to 90% (162). On a non-susceptible background, VISTA blockade also accelerates EAE onset and severity (159).

VISTA appears to function both as a negative receptor on T cells and as a ligand expressed on APCs interacting with an unknown receptor on T cells. As evidenced, in a passive transfer model of EAE, VISTA deficiency on both T cells and host contribute to the control of autoimmunity with the most aggressive disease obtained by transferring VISTA-deficient pathogenic T cells into VISTA-deficient hosts. VISTA expression on the host, however, appears to contribute more than that on T cells as VISTA-deficient hosts always present accelerated disease regardless of the status of the T cells transferred (163). *In vitro*, maximal antigen-specific proliferation is achieved when both APCs and T cells are deficient, again supporting that VISTA on both T cells and APCs contributes to the inhibition of T cell proliferation via specific pathways (161).

VISTA as a Ligand Negatively Regulates T Cell Activation

Several findings suggest that VISTA negatively regulates T cell responses by acting as a ligand that interacts with an unknown receptor on T cells. A soluble VISTA–Ig fusion protein inhibits

human and mouse CD4 T cell proliferation and cytokine production *in vitro* by suppressing early TCR activation (159, 160). A single dose of VISTA–Ig fusion protein prevents the development of GVHD in mice if given at the time of transplantation (158). When expressed on APCs, VISTA decreases antigen-specific T cell proliferation. VISTA blockade thus increases proliferation when T cells are stimulated with VISTA expressing myeloid APCs. Moreover, ectopic expression of VISTA on tumor cells interferes with protective anti-tumor immunity and allows increased tumor growth in vaccinated hosts (159).

VISTA as a Receptor Negatively Regulates T Cell Activation

Several findings also suggest a direct negative role of VISTA as a receptor on T cells. VISTA-deficient T cells respond by increased antigen-specific proliferation and cytokine production when compared to WT T cells *in vitro* and *in vivo*. On the other hand, VISTA engagement by an agonistic antibody suppresses antigen-specific proliferation when T cells are activated by VISTA-deficient APCs (161). In this system, because the APCs are deficient, VISTA on T cells functions independently of APCs to suppress T cell responses.

As a receptor on T cells, VISTA seems critical for the regulation of allogeneic responses. While an agonistic anti-VISTA antibody potently suppresses GVHD in mice (158), it does not prevent GVHD induced by VISTA-deficient donor T cells (164). In addition, VISTA-deficient T cells induce exacerbated GVHD with increased donor T cell expansion and decreased survival. However, VISTA-deficient recipient do not present aggravated GVHD, indicating that VISTA expression on recipient cells (APC) had little effect on the regulation of allogeneic T cells in this disease model (164).

VISTA Regulates Treg Differentiation and Suppressive Function

VISTA is also highly expressed by a subset of Tregs and like most NCRs seems to contribute to their differentiation and suppressive function. VISTA–Ig fusion protein promotes the induction of both human and murine Tregs *in vitro* (160, 165) and VISTA blockade decreases the generation of tumor-specific Tregs *in vivo* (165). This suggests that as a ligand, VISTA can promote Treg generation.

Some evidence suggests that VISTA may also be involved in the suppressive function of Tregs. First, VISTA expression is highly upregulated on tumor-infiltrating Tregs, indicating that VISTA on Tregs could play a role in suppressing tumor-specific immunity. In addition, VISTA blockade can reverse Treg-mediated suppression *in vitro*. However, VISTA blockade reverses suppression by both low VISTA expressing Tregs and high VISTA expressing Tregs and also increases the proliferation of T cells in the absence of Tregs, indicating that it might not be directly involved in Treg-mediated suppression. In this system, VISTA could function both as a receptor on T cells and a ligand expressed by Tregs (165).

VISTA Regulates Myeloid Cell Activation

As mentioned, VISTA is highly expressed by myeloid cells. Its expression can be further upregulated on human monocytes by TLR ligands as well as IL-10 and IFNγ. Overexpression of VISTA is observed in monocytes from chronically HIV-infected patients. Heightened VISTA expression correlates with immune activation and CD4 depletion (166). Enforced VISTA overexpression on human monocytes/macrophages induces spontaneous secretion of multiple inflammatory cytokines at levels similar to fully activated monocytes. The process requires signaling via VISTA as cytokine secretion is abrogated by deletion of the cytoplasmic

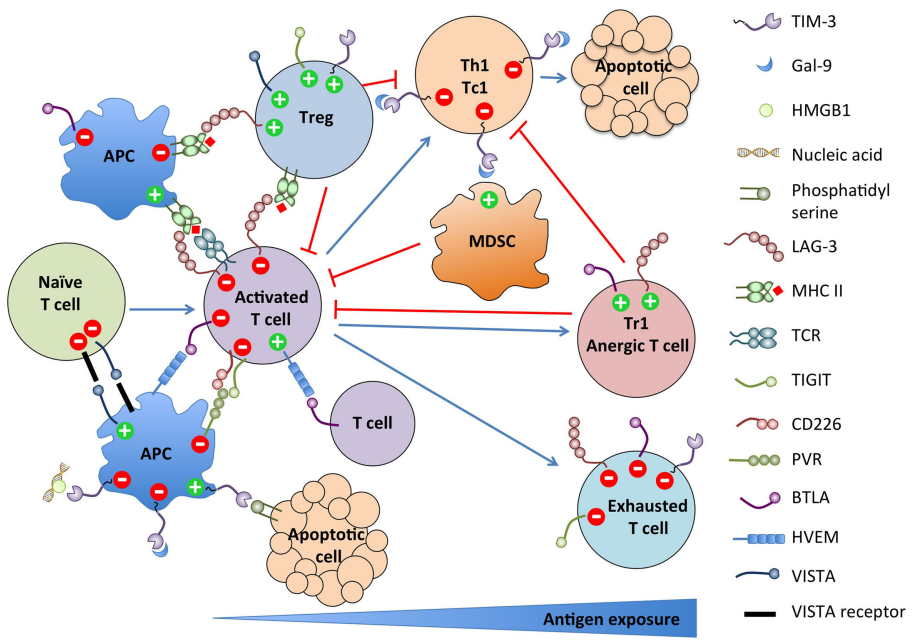

FIGURE 1 | Summary of NCRs expression on immune cell populations and their functions.

domain of VISTA. Interestingly, VISTA overexpression on HIV patients' monocytes also enhances their ability to stimulate IFNγ production by HIV-specific T cells. Furthermore, VISTA inactivation decreases the antigen presentation ability. In this pathological situation, VISTA positive regulation of myeloid cells seems to overcome the negative signal to T cells (166). Thus, in addition to acting as a ligand for T cells, VISTA acts as a receptor on myeloid cells to regulate their activation.

VISTA Negatively Regulates Anti-Tumor Immunity

VISTA is highly expressed on tumor-infiltrating leukocytes. Importantly, it is overexpressed on MDSCs and Tregs, suggesting that VISTA plays a role in tumor evasion from the immune system (165). Indeed, VISTA-deficient mice present elevated tumor-specific immune response and are more responsive to immunization against tumor antigens (163) as well as radiotherapy treatment (161). However, VISTA deficiency alone is not sufficient to reduce tumor growth (163).

VISTA blockade also alters the suppressive character of the tumor microenvironment, reducing MDSCs, increasing DC activation and enhancing the proliferation and the effector function of tumor-infiltrating CD4 and CD8 T cells. The enhanced tumor-specific immunity results in delayed tumor growth in multiple tumor models (165). However, as most monotherapies, anti-VISTA is not sufficient to lead to complete tumor rejection. When combined with a peptide vaccine, VISTA blockade shows synergistic efficacy leading to complete tumor eradication in an inducible melanoma model (165). In addition, anti-VISTA and anti-PD-L1 combination therapy leads to tumor regression and synergistically increases tumor-specific CD8 T cell effector functions in CT26 colon carcinoma model. In non-immunogenic tumor models, combination therapy synergizes with vaccination or Treg depletion to induce tumor rejection (162).

Concluding Remarks

As summarized in **Figure 1**, these new NCR pathways present striking similarities. Most NCRs are induced upon T cell activation and terminate or constrain the effector response by feedback inhibition. Some are also expressed on the APCs and regulate their stimulatory function. Conversely, multiple NCRs are expressed on Tregs and Tr1 and promote their differentiation and/or suppressive function. Most are also upregulated on dysfunctional T cells in chronic viral infections and cancer. Finally, most have multiple binding partners with which interactions are bidirectional with regard to signaling, rendering the assignment of ligand and receptor ambiguous or irrelevant. However, despite those similarities, their functions are mostly non-redundant. Therefore, blocking several of these pathways synergize in restoring efficient anti-tumor responses in preclinical models. The recently found astounding efficacy of combined anti-CTLA-4 and anti-PD-1 therapy in advanced melanoma patients argues in favor of targeting multiple pathways for future immunotherapeutic approaches (167–169).

References

1. Murakami N, Riella LV. Co-inhibitory pathways and their importance in immune regulation. *Transplantation* (2014) **98**(1):3–14. doi:10.1097/TP.0000000000000169
2. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* (2002) **415**(6871):536–41. doi:10.1038/415536a
3. Anderson AC, Lord GM, Dardalhon V, Lee DH, Sabatos-Peyton CA, Glimcher LH, et al. T-bet, a Th1 transcription factor regulates the expression of Tim-3. *Eur J Immunol* (2010) **40**(3):859–66. doi:10.1002/eji.200939842
4. Lee J, Su EW, Zhu C, Hainline S, Phuah J, Moroco JA, et al. Phosphotyrosine-dependent coupling of Tim-3 to T-cell receptor signaling pathways. *Mol Cell Biol* (2011) **31**(19):3963–74. doi:10.1128/MCB.05297-11
5. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* (2005) **6**(12):1245–52. doi:10.1038/ni1271
6. Asakura H, Kashio Y, Nakamura K, Seki M, Dai S, Shirato Y, et al. Selective eosinophil adhesion to fibroblast via IFN-gamma-induced galectin-9. *J Immunol* (2002) **169**(10):5912–8. doi:10.4049/jimmunol.169.10.5912
7. Sehrawat S, Reddy PB, Rajasagi N, Suryawanshi A, Hirashima M, Rouse BT. Galectin-9/TIM-3 interaction regulates virus-specific primary and memory CD8 T cell response. *PLoS Pathog* (2010) **6**(5):e1000882. doi:10.1371/journal.ppat.1000882
8. Boenisch O, D'Addio F, Watanabe T, Elyaman W, Magee CN, Yeung MY, et al. TIM-3: a novel regulatory molecule of alloimmune activation. *J Immunol* (2010) **185**(10):5806–19. doi:10.4049/jimmunol.0903435
9. Oomizu S, Arikawa T, Niki T, Kadowaki T, Ueno M, Nishi N, et al. Galectin-9 suppresses Th17 cell development in an IL-2-dependent but Tim-3-independent manner. *Clin Immunol* (2012) **143**(1):51–8. doi:10.1016/j.clim.2012.01.004
10. Lee SY, Goverman JM. The influence of T cell Ig mucin-3 signaling on central nervous system autoimmune disease is determined by the effector function of the pathogenic T cells. *J Immunol* (2013) **190**(10):4991–9. doi:10.4049/jimmunol.1300083
11. Sabatos CA, Chakravarti S, Cha E, Schubart A, Sánchez-Fueyo A, Zheng XX, et al. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. *Nat Immunol* (2003) **4**(11):1102–10. doi:10.1038/ni988
12. Sánchez-Fueyo A, Tian J, Picarella D, Domenig C, Zheng XX, Sabatos CA, et al. Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. *Nat Immunol* (2003) **4**(11):1093–101. doi:10.1038/ni987
13. Oikawa T, Kamimura Y, Akiba H, Yagita H, Okumura K, Takahashi H, et al. Preferential involvement of Tim-3 in the regulation of hepatic CD8+ T cells in murine acute graft-versus-host disease. *J Immunol* (2006) **177**(7):4281–7. doi:10.4049/jimmunol.177.7.4281
14. Veenstra RG, Taylor PA, Zhou Q, Panoskaltsis-Mortari A, Hirashima M, Flynn R, et al. Contrasting acute graft-versus-host disease effects of Tim-3/galectin-9 pathway blockade dependent upon the presence of donor regulatory T cells. *Blood* (2012) **120**(3):682–90. doi:10.1182/blood-2011-10-387977
15. Kearley J, McMillan SJ, Lloyd CM. Th2-driven, allergen-induced airway inflammation is reduced after treatment with anti-Tim-3 antibody in vivo. *J Exp Med* (2007) **204**(6):1289–94. doi:10.1084/jem.20062093
16. He W, Fang Z, Wang F, Wu K, Xu Y, Zhou H, et al. Galectin-9 significantly prolongs the survival of fully mismatched cardiac allografts in mice. *Transplantation* (2009) **88**(6):782–90. doi:10.1097/TP.0b013e3181b47f25
17. Wang F, He W, Zhou H, Yuan J, Wu K, Xu L, et al. The Tim-3 ligand galectin-9 negatively regulates CD8+ alloreactive T cell and prolongs survival of skin graft. *Cell Immunol* (2007) **250**(1–2):68–74. doi:10.1016/j.cellimm.2008.01.006
18. Wang F, Wan L, Zhang C, Zheng X, Li J, Chen ZK. Tim-3-galectin-9 pathway involves the suppression induced by CD4+CD25+ regulatory T cells. *Immunobiology* (2009) **214**(5):342–9. doi:10.1016/j.imbio.2008.10.007

19. Chou FC, Kuo CC, Wang YL, Lin MH, Linju Yen B, Chang DM, et al. Overexpression of galectin-9 in islets prolongs grafts survival via downregulation of Th1 responses. *Cell Transplant* (2013) **22**(11):2135–45. doi:10.3727/096368912X657891
20. Seki M, Oomizu S, Sakata KM, Sakata A, Arikawa T, Watanabe K, et al. Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. *Clin Immunol* (2008) **127**(1):78–88. doi:10.1016/j.clim.2008.01.006
21. Sehrawat S, Suryawanshi A, Hirashima M, Rouse BT. Role of Tim-3/galectin-9 inhibitory interaction in viral-induced immunopathology: shifting the balance toward regulators. *J Immunol* (2009) **182**(5):3191–201. doi:10.4049/jimmunol.0803673
22. Hastings WD, Anderson DE, Kassam N, Koguchi K, Greenfield EA, Kent SC, et al. TIM-3 is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines. *Eur J Immunol* (2009) **39**(9):2492–501. doi:10.1002/eji.200939274
23. Frisancho-Kiss S, Nyland JF, Davis SE, Barrett MA, Gatewood SJ, Njoku DB, et al. Cutting edge: T cell Ig mucin-3 reduces inflammatory heart disease by increasing CTLA-4 during innate immunity. *J Immunol* (2006) **176**(11):6411–5. doi:10.4049/jimmunol.176.11.6411
24. Yang X, Jiang X, Chen G, Xiao Y, Geng S, Kang C, et al. T cell Ig mucin-3 promotes homeostasis of sepsis by negatively regulating the TLR response. *J Immunol* (2013) **190**(5):2068–79. doi:10.4049/jimmunol.1202661
25. Frisancho-Kiss S, Davis SE, Nyland JF, Frisancho JA, Cihakova D, Barrett MA, et al. Cutting edge: cross-regulation by TLR4 and T cell Ig mucin-3 determines sex differences in inflammatory heart disease. *J Immunol* (2007) **178**(11):6710–4. doi:10.4049/jimmunol.178.11.6710
26. Zhang Y, Ma CJ, Wang JM, Ji XJ, Wu XY, Jia ZS, et al. Tim-3 negatively regulates IL-12 expression by monocytes in HCV infection. *PLoS One* (2011) **6**(5):e19664. doi:10.1371/journal.pone.0019664
27. Uchida Y, Ke B, Freitas MC, Yagita H, Akiba H, Busuttil RW, et al. T Cell Immunoglobulin mucin-3 dictates severity of liver ischemia/reperfusion injury in mice in TLR4-dependent manner. *Gastroenterology* (2010) **139**(6):2195–206. doi:10.1053/j.gastro.2010.07.003
28. Yan W, Liu X, Ma H, Zhang H, Song X, Gao L, et al. Tim-3 fosters HCC development by enhancing TGF-β-mediated alternative activation of macrophages. *Gut* (2015). doi:10.1136/gutjnl-2014-307671
29. Dai SY, Nakagawa R, Itoh A, Murakami H, Kashio Y, Abe H, et al. Galectin-9 induces maturation of human monocyte-derived dendritic cells. *J Immunol* (2005) **175**(5):2974–81. doi:10.4049/jimmunol.175.5.2974
30. Anderson AC, Anderson DE, Bregoli L, Hastings WD, Kassam N, Lei C, et al. Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells. *Science* (2007) **318**(5853):1141–3. doi:10.1126/science.1148536
31. Nagahara K, Arikawa T, Oomizu S, Kontani K, Nobumoto A, Tateno H, et al. Galectin-9 increases Tim-3+ dendritic cells and CD8+ T cells and enhances antitumor immunity via galectin-9-Tim-3 interactions. *J Immunol* (2008) **181**(11):7660–9. doi:10.4049/jimmunol.181.11.7660
32. Jayaraman P, Sada-Ovalle I, Beladi S, Anderson AC, Dardalhon V, Hotta C, et al. Tim3 binding to galectin-9 stimulates antimicrobial immunity. *J Exp Med* (2010) **207**(11):2343–54. doi:10.1084/jem.20100687
33. Sada-Ovalle I, Chávez-Galán L, Torre-Bouscoulet L, Nava-Gamiño L, Barrera L, Jayaraman P, et al. The Tim3-galectin 9 pathway induces antibacterial activity in human macrophages infected with *M. tuberculosis*. *J Immunol* (2012) **189**(12):5896–902. doi:10.4049/jimmunol.1200990
34. Dardalhon V, Anderson AC, Karman J, Apetoh L, Chandwaskar R, Lee DH, et al. Tim-3/galectin-9 pathway: regulation of Th1 immunity through promotion of CD11b+*Ly-6G*+ myeloid cells. *J Immunol* (2010) **185**(3):1383–92. doi:10.4049/jimmunol.0903275
35. Ma CJ, Li GY, Cheng YQ, Wang JM, Ying RS, Shi L, et al. Cis association of galectin-9 with Tim-3 differentially regulates IL-12/IL-23 expressions in monocytes via TLR signaling. *PLoS One* (2013) **8**(8):e72488. doi:10.1371/journal.pone.0072488
36. Su EW, Bi S, Kane LP. Galectin-9 regulates T helper cell function independently of Tim-3. *Glycobiology* (2011) **21**(10):1258–65. doi:10.1093/glycob/cwq214
37. Moritoki M, Kadowaki T, Niki T, Nakano D, Soma G, Mori H, et al. Galectin-9 ameliorates clinical severity of MRL/lpr lupus-prone mice by inducing plasma cell apoptosis independently of Tim-3. *PLoS One* (2013) **8**(4):e60807. doi:10.1371/journal.pone.0060807
38. Leitner J, Rieger A, Pickl WF, Zlabinger G, Grabmeier-Pfistershammer K, Steinberger P. TIM-3 does not act as a receptor for galectin-9. *PLoS Pathog* (2013) **9**(3):e1003253. doi:10.1371/journal.ppat.1003253
39. Cao E, Zang X, Ramagopal UA, Mukhopadhaya A, Fedorov A, Fedorov E, et al. T cell immunoglobulin mucin-3 crystal structure reveals a galectin-9-independent ligand-binding surface. *Immunity* (2007) **26**(3):311–21. doi:10.1016/j.immuni.2007.01.016
40. DeKruyff RH, Bu X, Ballesteros A, Santiago C, Chim YL, Lee HH, et al. T cell/transmembrane, Ig, and mucin-3 allelic variants differentially recognize phosphatidylserine and mediate phagocytosis of apoptotic cells. *J Immunol* (2010) **184**(4):1918–30. doi:10.4049/jimmunol.0903059
41. Nakayama M, Akiba H, Takeda K, Kojima Y, Hashiguchi M, Azuma M, et al. Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation. *Blood* (2009) **113**(16):3821–30. doi:10.1182/blood-2008-10-185884
42. Chabtini L, Mfarrej B, Mounayar M, Zhu B, Batal I, Dakle PJ, et al. TIM-3 regulates innate immune cells to induce fetomaternal tolerance. *J Immunol* (2013) **190**(1):88–96. doi:10.4049/jimmunol.1202176
43. Chiba S, Baghdadi M, Akiba H, Yoshiyama H, Kinoshita I, Dosaka-Akita H, et al. Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. *Nat Immunol* (2012) **13**(9):832–42. doi:10.1038/ni.2376
44. Huang YH, Zhu C, Kondo Y, Anderson AC, Gandhi A, Russell A, et al. CEACAM1 regulates TIM-3-mediated tolerance and exhaustion. *Nature* (2015) **517**(7534):386–90. doi:10.1038/nature13848
45. Golden-Mason L, Palmer BE, Kassam N, Townshend-Bulson L, Livingston S, McMahon BJ, et al. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol* (2009) **83**(18):9122–30. doi:10.1128/JVI.00639-09
46. Jin HT, Anderson AC, Tan WG, West EE, Ha SJ, Araki K, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A* (2010) **107**(33):14733–8. doi:10.1073/pnas.1009731107
47. Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, Long BR, et al. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med* (2008) **205**(12):2763–79. doi:10.1084/jem.20081398
48. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med* (2010) **207**(10):2187–94. doi:10.1084/jem.20100643
49. Zhou Q, Munger ME, Veenstra RG, Weigel BJ, Hirashima M, Munn DH, et al. Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. *Blood* (2011) **117**(17):4501–10. doi:10.1182/blood-2010-10-310425
50. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med* (2010) **207**(10):2175–86. doi:10.1084/jem.20100637
51. Yang ZZ, Grote DM, Ziesmer SC, Niki T, Hirashima M, Novak AJ, et al. IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. *J Clin Invest* (2012) **122**(4):1271–82. doi:10.1172/JCI59806
52. Takamura S, Tsuji-Kawahara S, Yagita H, Akiba H, Sakamoto M, Chikaishi T, et al. Premature terminal exhaustion of friend virus-specific effector CD8+ T cells by rapid induction of multiple inhibitory receptors. *J Immunol* (2010) **184**(9):4696–707. doi:10.4049/jimmunol.0903478
53. Gao X, Zhu Y, Li G, Huang H, Zhang G, Wang F, et al. TIM-3 expression characterizes regulatory T cells in tumor tissues and is associated with lung cancer progression. *PLoS One* (2012) **7**(2):e30676. doi:10.1371/journal.pone.0030676
54. Sakuishi K, Ngiow SF, Sullivan JM, Teng MW, Kuchroo VK, Smyth MJ, et al. TIM3FOXP3 regulatory T cells are tissue-specific promoters of T-cell dysfunction in cancer. *Oncoimmunology* (2013) **2**(4):e23849. doi:10.4161/onci.23849

55. Yan J, Zhang Y, Zhang JP, Liang J, Li L, Zheng L. Tim-3 expression defines regulatory T cells in human tumors. *PLoS One* (2013) **8**(3):e58006. doi:10.1371/journal.pone.0058006
56. Triebel F, Jitsukawa S, Baixeras E, Roman-Roman S, Genevee C, Viegas-Pequignot E, et al. LAG-3, a novel lymphocyte activation gene closely related to CD4. *J Exp Med* (1990) **171**(5):1393–405. doi:10.1084/jem.171.5.1393
57. Huard B, Prigent P, Tournier M, Bruniquel D, Triebel F. CD4/major histocompatibility complex class II interaction analyzed with CD4- and lymphocyte activation gene-3 (LAG-3)-Ig fusion proteins. *Eur J Immunol* (1995) **25**(9):2718–21. doi:10.1002/eji.1830250949
58. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in regulatory T cells. *Immunity* (2004) **21**(4):503–13. doi:10.1016/j.immuni.2004.08.010
59. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med* (2013) **19**(6):739–46. doi:10.1038/nm.3179
60. Okamura T, Fujio K, Shibuya M, Sumitomo S, Shoda H, Sakaguchi S, et al. CD4(+)CD25(−)LAG3(+) regulatory T cells controlled by the transcription factor Egr-2. *Proc Natl Acad Sci USA* (2009) **106**(33):13974–9. doi:10.1073/pnas.0906872106
61. Baixeras E, Huard B, Miossec C, Jitsukawa S, Martin M, Hercend T, et al. Characterization of the lymphocyte activation gene 3-encoded protein. A new ligand for human leukocyte antigen class II antigens. *J Exp Med* (1992) **176**(2):327–37. doi:10.1084/jem.176.2.327
62. Kisielow M, Kisielow J, Capoferri-Sollami G, Karjalainen K. Expression of lymphocyte activation gene 3 (LAG-3) on B cells is induced by T cells. *Eur J Immunol* (2005) **35**(7):2081–8. doi:10.1002/eji.200526090
63. Workman CJ, Wang Y, El Kasmi KC, Pardoll DM, Murray PJ, Drake CG, et al. LAG-3 regulates plasmacytoid dendritic cell homeostasis. *J Immunol* (2009) **182**(4):1885–91. doi:10.4049/jimmunol.0800185
64. Bae J, Lee SJ, Park CG, Lee YS, Chun T. Trafficking of LAG-3 to the surface on activated T cells via its cytoplasmic domain and protein kinase C signaling. *J Immunol* (2014) **193**(6):3101–12. doi:10.4049/jimmunol.1401025
65. Woo SR, Li N, Bruno TC, Forbes K, Brown S, Workman C, et al. Differential subcellular localization of the regulatory T-cell protein LAG-3 and the coreceptor CD4. *Eur J Immunol* (2010) **40**(6):1768–77. doi:10.1002/eji.200939874
66. Li N, Wang Y, Forbes K, Vignali KM, Heale BS, Saftig P, et al. Metalloproteases regulate T-cell proliferation and effector function via LAG-3. *EMBO J* (2007) **26**(2):494–504. doi:10.1038/sj.emboj.7601520
67. Grosso JF, Goldberg MV, Getnet D, Bruno TC, Yen HR, Pyle KJ, et al. Functionally distinct LAG-3 and PD-1 subsets on activated and chronically stimulated CD8 T cells. *J Immunol* (2009) **182**(11):6659–69. doi:10.4049/jimmunol.0804211
68. Hannier S, Triebel F. The MHC class II ligand lymphocyte activation gene-3 is co-distributed with CD8 and CD3-TCR molecules after their engagement by mAb or peptide-MHC class I complexes. *Int Immunol* (1999) **11**(11):1745–52. doi:10.1093/intimm/11.11.1745
69. Workman CJ, Dugger KJ, Vignali DAA. Cutting edge: molecular analysis of the negative regulatory function of lymphocyte activation gene-3. *J Immunol* (2002) **169**(10):5392–5. doi:10.4049/jimmunol.169.10.5392
70. Miyazaki T, Dierich A, Benoist C, Mathis D. Independent modes of natural killing distinguished in mice lacking Lag3. *Science* (1996) **272**(5260):405–8. doi:10.1126/science.272.5260.405
71. Okazaki T, Okazaki IM, Wang J, Sugiura D, Nakaki F, Yoshida T, et al. PD-1 and LAG-3 inhibitory co-receptors act synergistically to prevent autoimmunity in mice. *J Exp Med* (2011) **208**(2):395–407. doi:10.1084/jem.20100466
72. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res* (2012) **72**(4):917–27. doi:10.1158/0008-5472.CAN-11-1620
73. Bettini M, Szymczak-Workman AL, Forbes K, Castellaw AH, Selby M, Pan X, et al. Cutting edge: accelerated autoimmune diabetes in the absence of LAG-3. *J Immunol* (2011) **187**(7):3493–8. doi:10.4049/jimmunol.1100714
74. Huard B, Gaulard P, Faure F, Hercend T, Triebel F. Cellular expression and tissue distribution of the human LAG-3-encoded protein, an MHC class II ligand. *Immunogenetics* (1994) **39**(3):213–7. doi:10.1007/BF00241263
75. Macon-Lemaitre L, Triebel F. The negative regulatory function of the lymphocyte-activation gene-3 co-receptor (CD223) on human T cells. *Immunology* (2005) **115**(2):170–8. doi:10.1111/j.1365-2567.2005.02145.x
76. Workman CJ, Cauley LS, Kim IJ, Blackman MA, Woodland DL, Vignali DA. Lymphocyte activation gene-3 (CD223) regulates the size of the expanding T cell population following antigen activation in vivo. *J Immunol* (2004) **172**(9):5450–5. doi:10.4049/jimmunol.172.9.5450
77. Sega EI, Leveson-Gower DB, Florek M, Schneidawind D, Luong RH, Negrin RS. Role of lymphocyte activation gene-3 (Lag-3) in conventional and regulatory T cell function in allogeneic transplantation. *PLoS One* (2014) **9**(1):e86551. doi:10.1371/journal.pone.0086551
78. Grosso JF, Kelleher CC, Harris TJ, Maris CH, Hipkiss EL, De Marzo A, et al. LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. *J Clin Invest* (2007) **117**(11):3383–92. doi:10.1172/JCI31184
79. Lucas CL, Workman CJ, Beyaz S, LoCascio S, Zhao G, Vignali DA, et al. LAG-3, TGF-beta, and cell-intrinsic PD-1 inhibitory pathways contribute to CD8 but not CD4 T-cell tolerance induced by allogeneic BMT with anti-CD40L. *Blood* (2011) **117**(20):5532–40. doi:10.1182/blood-2010-11-318675
80. Hannier S, Tournier M, Bismuth G, Triebel F. CD3/TCR complex-associated lymphocyte activation gene-3 molecules inhibit CD3/TCR signaling. *J Immunol* (1998) **161**(8):4058–65.
81. Durham NM, Nirschl CJ, Jackson CM, Elias J, Kochel CM, Anders RA, et al. Lymphocyte activation gene 3 (LAG-3) modulates the ability of CD4 T-cells to be suppressed in vivo. *PLoS One* (2014) **9**(11):e109080. doi:10.1371/journal.pone.0109080
82. Workman CJ, Vignali DA. Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). *J Immunol* (2005) **174**(2):688–95. doi:10.4049/jimmunol.174.2.688
83. Avice MN, Sarfati M, Triebel F, Delespesse G, Demeure CE. Lymphocyte activation gene-3, a MHC class II ligand expressed on activated T cells, stimulates TNF-alpha and IL-12 production by monocytes and dendritic cells. *J Immunol* (1999) **162**(5):2748–53.
84. Andreae S, Piras F, Burdin N, Triebel F. Maturation and activation of dendritic cells induced by lymphocyte activation gene-3 (CD223). *J Immunol* (2002) **168**(8):3874–80. doi:10.4049/jimmunol.168.8.3874
85. El Mir S, Triebel F. A soluble lymphocyte activation gene-3 molecule used as a vaccine adjuvant elicits greater humoral and cellular immune responses to both particulate and soluble antigens. *J Immunol* (2000) **164**(11):5583–9. doi:10.4049/jimmunol.164.11.5583
86. Prigent P, El Mir S, Dréano M, Triebel F. Lymphocyte activation gene-3 induces tumor regression and antitumor immune responses. *Eur J Immunol* (1999) **29**(12):3867–76. doi:10.1002/(SICI)1521-4141(199912)29:12<3867::AID-IMMU3867>3.3.CO;2-5
87. Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol* (2008) **180**(9):5916–26. doi:10.4049/jimmunol.180.9.5916
88. Andreae S, Buisson S, Triebel F. MHC class II signal transduction in human dendritic cells induced by a natural ligand, the LAG-3 protein (CD223). *Blood* (2003) **102**(6):2130–7. doi:10.1182/blood-2003-01-0273
89. Xu F, Liu J, Liu D, Liu B, Wang M, Hu Z, et al. LSECtin expressed on melanoma cells promotes tumor progression by inhibiting antitumor T-cell responses. *Cancer Res* (2014) **74**(13):3418–28. doi:10.1158/0008-5472.CAN-13-2690
90. Kouo T, Huang L, Pucsek AB, Cao M, Solt S, Armstrong T, et al. Galectin-3 shapes antitumor immune responses by suppressing CD8+ T cells via LAG-3 and inhibiting expansion of plasmacytoid dendritic cells. *Cancer Immunol Res* (2015) **3**(4):412–23. doi:10.1158/2326-6066.CIR-14-0150
91. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* (2009) **10**(1):29–37. doi:10.1038/ni.1679
92. Tian X, Zhang A, Qiu C, Wang W, Yang Y, Qiu C, et al. The upregulation of LAG-3 on T cells defines a subpopulation with functional exhaustion and correlates with disease progression in HIV-infected subjects. *J Immunol* (2015) **194**(8):3873–82. doi:10.4049/jimmunol.1402176
93. Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, Beck A, Miller A, Tsuji T, et al. Tumor-infiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. *Proc Natl Acad Sci U S A* (2010) **107**(17):7875–80. doi:10.1073/pnas.1003345107
94. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, et al. The surface protein TIGIT suppresses T cell activation by promoting the

generation of mature immunoregulatory dendritic cells. *Nat Immunol* (2009) **10**(1):48–57. doi:10.1038/ni.1674
95. Stanietsky N, Simic H, Arapovic J, Toporik A, Levy O, Novik A, et al. The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proc Natl Acad Sci U S A* (2009) **106**(42):17858–63. doi:10.1073/pnas.0903474106
96. Levin SD, Taft DW, Brandt CS, Bucher C, Howard ED, Chadwick EM, et al. Vstm3 is a member of the CD28 family and an important modulator of T-cell function. *Eur J Immunol* (2011) **41**(4):902–15. doi:10.1002/eji.201041136
97. Carlsten M, Björkström NK, Norell H, Bryceson Y, van Hall T, Baumann BC, et al. DNAX accessory molecule-1 mediated recognition of freshly isolated ovarian carcinoma by resting natural killer cells. *Cancer Res* (2007) **67**(3):1317–25. doi:10.1158/0008-5472.CAN-06-2264
98. Masson D, Jarry A, Baury B, Blanchardie P, Laboisse C, Lustenberger P, et al. Overexpression of the CD155 gene in human colorectal carcinoma. *Gut* (2001) **49**(2):236–40. doi:10.1136/gut.49.2.236
99. Hirota T, Irie K, Okamoto R, Ikeda W, Takai Y. Transcriptional activation of the mouse Necl-5/Tage4/PVR/CD155 gene by fibroblast growth factor or oncogenic Ras through the Raf-MEK-ERK-AP-1 pathway. *Oncogene* (2005) **24**(13):2229–35. doi:10.1038/sj.onc.1208409
100. Soriani A, Zingoni A, Cerboni C, Iannitto ML, Ricciardi MR, Di Gialleonardo V, et al. ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. *Blood* (2009) **113**(15):3503–11. doi:10.1182/blood-2008-08-173914
101. Kamran N, Takai Y, Miyoshi J, Biswas SK, Wong JS, Gasser S. Toll-like receptor ligands induce expression of the costimulatory molecule CD155 on antigen-presenting cells. *PLoS One* (2013) **8**(1):e54406. doi:10.1371/journal.pone.0054406
102. Dardalhon V, Schubart AS, Reddy J, Meyers JH, Monney L, Sabatos CA, et al. CD226 is specifically expressed on the surface of Th1 cells and regulates their expansion and effector functions. *J Immunol* (2005) **175**(3):1558–65. doi:10.4049/jimmunol.175.3.1558
103. Li M, Xia P, Du Y, Liu S, Huang G, Chen J, et al. T-cell immunoglobulin and ITIM domain (TIGIT) receptor/poliovirus receptor (PVR) ligand engagement suppresses interferon-gamma production of natural killer cells via beta-arrestin 2-mediated negative signaling. *J Biol Chem* (2014) **289**(25):17647–57. doi:10.1074/jbc.M114.572420
104. Liu S, Zhang H, Li M, Hu D, Li C, Ge B, et al. Recruitment of Grb2 and SHIP1 by the ITT-like motif of TIGIT suppresses granule polarization and cytotoxicity of NK cells. *Cell Death Differ* (2013) **20**(3):456–64. doi:10.1038/cdd.2012.141
105. Stanietsky N, Rovis TL, Glasner A, Seidel E, Tsukerman P, Yamin R, et al. Mouse TIGIT inhibits NK-cell cytotoxicity upon interaction with PVR. *Eur J Immunol* (2013) **43**(8):2138–50. doi:10.1002/eji.201243072
106. Tahara-Hanaoka S, Shibuya K, Kai H, Miyamoto A, Morikawa Y, Ohkochi N, et al. Tumor rejection by the poliovirus receptor family ligands of the DNAM-1 (CD226) receptor. *Blood* (2006) **107**(4):1491–6. doi:10.1182/blood-2005-04-1684
107. Chan CJ, Andrews DM, McLaughlin NM, Yagita H, Gilfillan S, Colonna M, et al. DNAM-1/CD155 interactions promote cytokine and NK cell-mediated suppression of poorly immunogenic melanoma metastases. *J Immunol* (2010) **184**(2):902–11. doi:10.4049/jimmunol.0903225
108. Gilfillan S, Chan CJ, Cella M, Haynes NM, Rapaport AS, Boles KS, et al. DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. *J Exp Med* (2008) **205**(13):2965–73. doi:10.1084/jem.20081752
109. Welch MJ, Teijaro JR, Lewicki HA, Colonna M, Oldstone MB. CD8 T cell defect of TNF-alpha and IL-2 in DNAM-1 deficient mice delays clearance in vivo of a persistent virus infection. *Virology* (2012) **429**(2):163–70. doi:10.1016/j.virol.2012.04.006
110. Iguchi-Manaka A, Kai H, Yamashita Y, Shibata K, Tahara-Hanaoka S, Honda S, et al. Accelerated tumor growth in mice deficient in DNAM-1 receptor. *J Exp Med* (2008) **205**(13):2959–64. doi:10.1084/jem.20081611
111. Shirakawa J, Wang Y, Tahara-Hanaoka S, Honda S, Shibuya K, Shibuya A. LFA-1-dependent lipid raft recruitment of DNAM-1 (CD226) in CD4+ T cell. *Int Immunol* (2006) **18**(6):951–7. doi:10.1093/intimm/dxl031
112. Shirakawa J, Shibuya K, Shibuya A. Requirement of the serine at residue 329 for lipid raft recruitment of DNAM-1 (CD226). *Int Immunol* (2005) **17**(3):217–23. doi:10.1093/intimm/dxh199
113. Shibuya K, Shirakawa J, Kameyama T, Honda S, Tahara-Hanaoka S, Miyamoto A, et al. CD226 (DNAM-1) is involved in lymphocyte function-associated antigen 1 costimulatory signal for naive T cell differentiation and proliferation. *J Exp Med* (2003) **198**(12):1829–39. doi:10.1084/jem.20030958
114. Shibuya K, Lanier LL, Phillips JH, Ochs HD, Shimizu K, Nakayama E, et al. Physical and functional association of LFA-1 with DNAM-1 adhesion molecule. *Immunity* (1999) **11**(5):615–23. doi:10.1016/S1074-7613(00)80136-3
115. Ramsbottom KM, Hawkins ED, Shimoni R, McGrath M, Chan CJ, Russell SM, et al. Cutting edge: DNAX accessory molecule 1-deficient CD8+ T cells display immunological synapse defects that impair antitumor immunity. *J Immunol* (2014) **192**(2):553–7. doi:10.4049/jimmunol.1302197
116. Maiti AK, Kim-Howard X, Viswanathan P, Guillén L, Qian X, Rojas-Villarraga A, et al. Non-synonymous variant (Gly307Ser) in CD226 is associated with susceptibility to multiple autoimmune diseases. *Rheumatology (Oxford)* (2010) **49**(7):1239–44. doi:10.1093/rheumatology/kep470
117. Hafler JP, Maier LM, Cooper JD, Plagnol V, Hinks A, Simmonds MJ, et al. CD226 Gly307Ser association with multiple autoimmune diseases. *Genes Immun* (2009) **10**(1):5–10. doi:10.1038/gene.2008.82
118. Joller N, Hafler JP, Brynedal B, Kassam N, Spoerl S, Levin SD, et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol* (2011) **186**(3):1338–42. doi:10.4049/jimmunol.1003081
119. Zhang T, Wang J, Zhou X, Liang R, Bai Q, Yang L, et al. Increased expression of TIGIT on CD4+ T cells ameliorates immune-mediated bone marrow failure of aplastic anemia. *J Cell Biochem* (2014) **115**(11):1918–27. doi:10.1002/jcb.24862
120. Lozano E, Dominguez-Villar M, Kuchroo V, Hafler DA. The TIGIT/CD226 axis regulates human T cell function. *J Immunol* (2012) **188**(8):3869–75. doi:10.4049/jimmunol.1103627
121. Johnston RJ, Comps-Agrar L, Hackney J, Yu X, Huseni M, Yang Y, et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell* (2014) **26**(6):923–37. doi:10.1016/j.ccell.2014.10.018
122. Chan CJ, Martinet L, Gilfillan S, Souza-Fonseca-Guimaraes F, Chow MT, Town L, et al. The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. *Nat Immunol* (2014) **15**(5):431–8. doi:10.1038/ni.2850
123. Zhang Y, Maksimovic J, Naselli G, Qian J, Chopin M, Blewitt ME, et al. Genome-wide DNA methylation analysis identifies hypomethylated genes regulated by FOXP3 in human regulatory T cells. *Blood* (2013) **122**(16):2823–36. doi:10.1182/blood-2013-02-481788
124. Joller N, Lozano E, Burkett PR, Patel B, Xiao S, Zhu C, et al. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* (2014) **40**(4):569–81. doi:10.1016/j.immuni.2014.02.012
125. Fuhrman CA, Yeh WI, Seay HR, Saikumar Lakshmi P, Chopra G, Zhang L, et al. Divergent phenotypes of human regulatory T cells expressing the receptors TIGIT and CD226. *J Immunol* (2015) **195**(1):145–55. doi:10.4049/jimmunol.1402381
126. Bin Dhuban K, d'Hennezel E, Nashi E, Bar-Or A, Rieder S, Shevach EM, et al. Coexpression of TIGIT and FCRL3 identifies Helios+ human memory regulatory T cells. *J Immunol* (2015) **194**(8):3687–96. doi:10.4049/jimmunol.1401803
127. Cella M, Presti R, Vermi W, Lavender K, Turnbull E, Ochsenbauer-Jambor C, et al. Loss of DNAM-1 contributes to CD8+ T-cell exhaustion in chronic HIV-1 infection. *Eur J Immunol* (2010) **40**(4):949–54. doi:10.1002/eji.200940234
128. Han P, Goularte OD, Rufner K, Wilkinson B, Kaye J. An inhibitory Ig superfamily protein expressed by lymphocytes and APCs is also an early marker of thymocyte positive selection. *J Immunol* (2004) **172**(10):5931–9. doi:10.4049/jimmunol.172.10.5931
129. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol* (2003) **4**(7):670–9. doi:10.1038/ni944

130. Hurchla MA, Sedy JR, Gavrieli M, Drake CG, Murphy TL, Murphy KM. B and T lymphocyte attenuator exhibits structural and expression polymorphisms and is highly Induced in anergic CD4+ T cells. *J Immunol* (2005) **174**(6):3377–85. doi:10.4049/jimmunol.174.9.5884a
131. Legat A, Speiser DE, Pircher H, Zehn D, Fuertes Marraco SA. Inhibitory receptor expression depends more dominantly on differentiation and activation than "exhaustion" of human CD8 T cells. *Front Immunol* (2013) **4**:455. doi:10.3389/fimmu.2013.00455
132. Gonzalez LC, Loyet KM, Calemine-Fenaux J, Chauhan V, Wranik B, Ouyang W, et al. A coreceptor interaction between the CD28 and TNF receptor family members B and T lymphocyte attenuator and herpesvirus entry mediator. *Proc Natl Acad Sci U S A* (2005) **102**(4):1116–21. doi:10.1073/pnas.0409071102
133. Sedy JR, Gavrieli M, Potter KG, Hurchla MA, Lindsley RC, Hildner K, et al. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. *Nat Immunol* (2005) **6**(1):90–8. doi:10.1038/ni1144
134. Cai G, Anumanthan A, Brown JA, Greenfield EA, Zhu B, Freeman GJ. CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. *Nat Immunol* (2008) **9**(2):176–85. doi:10.1038/ni1554
135. Krieg C, Han P, Stone R, Goularte OD, Kaye J. Functional analysis of B and T lymphocyte attenuator engagement on CD4+ and CD8+ T cells. *J Immunol* (2005) **175**(10):6420–7. doi:10.4049/jimmunol.175.10.6420
136. Oya Y, Watanabe N, Owada T, Oki M, Hirose K, Suto A, et al. Development of autoimmune hepatitis-like disease and production of autoantibodies to nuclear antigens in mice lacking B and T lymphocyte attenuator. *Arthritis Rheum* (2008) **58**(8):2498–510. doi:10.1002/art.23674
137. Liu X, Alexiou M, Martin-Orozco N, Chung Y, Nurieva RI, Ma L, et al. Cutting edge: a critical role of B and T lymphocyte attenuator in peripheral T cell tolerance induction. *J Immunol* (2009) **182**(8):4516–20. doi:10.4049/jimmunol.0803161
138. Wang Y, Subudhi SK, Anders RA, Lo J, Sun Y, Blink S, et al. The role of herpesvirus entry mediator as a negative regulator of T cell-mediated responses. *J Clin Invest* (2005) **115**(3):711–7. doi:10.1172/JCI200522982
139. Miller ML, Sun Y, Fu YX. Cutting edge: B and T lymphocyte attenuator signaling on NKT cells inhibits cytokine release and tissue injury in early immune responses. *J Immunol* (2009) **183**(1):32–6. doi:10.4049/jimmunol.0900690
140. Uchiyama M, Jin X, Matsuda H, Bashuda H, Imazuru T, Shimokawa T, et al. An agonistic anti-BTLA mAb (3C10) induced generation of IL-10-dependent regulatory CD4+ T cells and prolongation of murine cardiac allograft. *Transplantation* (2014) **97**(3):301–9. doi:10.1097/01.TP.0000438204.96723.8b
141. Albring JC, Sandau MM, Rapaport AS, Edelson BT, Satpathy A, Mashayekhi M, et al. Targeting of B and T lymphocyte associated (BTLA) prevents graft-versus-host disease without global immunosuppression. *J Exp Med* (2010) **207**(12):2551–9. doi:10.1084/jem.20102017
142. Yang X, Zhang X, Sun Y, Tu T, Fu ML, Miller M, et al. A BTLA-mediated bait and switch strategy permits Listeria expansion in CD8alpha(+) DCs to promote long-term T cell responses. *Cell Host Microbe* (2014) **16**(1):68–80. doi:10.1016/j.chom.2014.05.021
143. Bekiaris V, Sedý JR, Macauley MG, Rhode-Kurnow A, Ware CF. The inhibitory receptor BTLA controls gammadelta T cell homeostasis and inflammatory responses. *Immunity* (2013) **39**(6):1082–94. doi:10.1016/j.immuni.2013.10.017
144. Gertner-Dardenne J, Fauriat C, Orlanducci F, Thibult ML, Pastor S, Fitzgibbon J, et al. The co-receptor BTLA negatively regulates human Vgamma9Vdelta2 T-cell proliferation: a potential way of immune escape for lymphoma cells. *Blood* (2013) **122**(6):922–31. doi:10.1182/blood-2012-11-464685
145. Steinberg MW, Turovskaya O, Shaikh RB, Kim G, McCole DF, Pfeffer K, et al. A crucial role for HVEM and BTLA in preventing intestinal inflammation. *J Exp Med* (2008) **205**(6):1463–76. doi:10.1084/jem.20071160
146. Deppong C, Degnan JM, Murphy TL, Murphy KM, Green JM. B and T lymphocyte attenuator regulates T cell survival in the lung. *J Immunol* (2008) **181**(5):2973–9. doi:10.4049/jimmunol.181.5.2973
147. Flynn R, Hutchinson T, Murphy KM, Ware CF, Croft M, Salek-Ardakani S. CD8 T cell memory to a viral pathogen requires trans cosignaling between HVEM and BTLA. *PLoS One* (2013) **8**(10):e77991. doi:10.1371/journal.pone.0077991
148. Hurchla MA, Sedy JR, Murphy KM. Unexpected role of B and T lymphocyte attenuator in sustaining cell survival during chronic allostimulation. *J Immunol* (2007) **178**(10):6073–82. doi:10.4049/jimmunol.178.10.6073
149. Zeng JC, Lin DZ, Yi LL, Liu GB, Zhang H, Wang WD, et al. BTLA exhibits immune memory for αβ T cells in patients with active pulmonary tuberculosis. *Am J Transl Res* (2014) **6**(5):494–506.
150. Sakoda Y, Park JJ, Zhao Y, Kuramasu A, Geng D, Liu Y, et al. Dichotomous regulation of GVHD through bidirectional functions of the BTLA-HVEM pathway. *Blood* (2011) **117**(8):2506–14. doi:10.1182/blood-2010-08-301325
151. Cheung TC, Steinberg MW, Oborne LM, Macauley MG, Fukuyama S, Sanjo H, et al. Unconventional ligand activation of herpesvirus entry mediator signals cell survival. *Proc Natl Acad Sci U S A* (2009) **106**(15):6244–9. doi:10.1073/pnas.0902115106
152. Cheung TC, Oborne LM, Steinberg MW, Macauley MG, Fukuyama S, Sanjo H, et al. T cell intrinsic heterodimeric complexes between HVEM and BTLA determine receptivity to the surrounding microenvironment. *J Immunol* (2009) **183**(11):7286–96. doi:10.4049/jimmunol.0902490
153. Derré L, Rivals JP, Jandus C, Pastor S, Rimoldi D, Romero P, et al. BTLA mediates inhibition of human tumor-specific CD8+ T cells that can be partially reversed by vaccination. *J Clin Invest* (2010) **120**(1):157–67. doi:10.1172/JCI40070
154. Fourcade J, Sun Z, Pagliano O, Guillaume P, Luescher IF, Sander C, et al. CD8(+) T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1. *Cancer Res* (2012) **72**(4):887–96. doi:10.1158/0008-5472.CAN-11-2637
155. Han L, Wang W, Fang Y, Feng Z, Liao S, Li W, et al. Soluble B and T lymphocyte attenuator possesses antitumor effects and facilitates heat shock protein 70 vaccine-triggered antitumor immunity against a murine TC-1 cervical cancer model in vivo. *J Immunol* (2009) **183**(12):7842–50. doi:10.4049/jimmunol.0804379
156. Lasaro MO, Sazanovich M, Giles-Davis W, Mrass P, Bunte RM, Sewell DA, et al. Active immunotherapy combined with blockade of a coinhibitory pathway achieves regression of large tumor masses in cancer-prone mice. *Mol Ther* (2011) **19**(9):1727–36. doi:10.1038/mt.2011.88
157. Haymaker C, Wu R, Ritthipichai K, Bernatchez C, Forget MA, Chen JQ, et al. Uncovering a novel function of BTLA on tumor-infiltrating CD8+ T cells. *J Immunother Cancer* (2013) **1**(Suppl 1):O1. doi:10.1186/2051-1426-1-S1-O1
158. Flies DB, Wang S, Xu H, Chen L. Cutting edge: a monoclonal antibody specific for the programmed death-1 homolog prevents graft-versus-host disease in mouse models. *J Immunol* (2011) **187**(4):1537–41. doi:10.4049/jimmunol.1100660
159. Wang L, Rubinstein R, Lines JL, Wasiuk A, Ahonen C, Guo Y, et al. VISTA, a novel mouse Ig superfamily ligand that negatively regulates T cell responses. *J Exp Med* (2011) **208**(3):577–92. doi:10.1084/jem.20100619
160. Lines JL, Pantazi E, Mak J, Sempere LF, Wang L, O'Connell S, et al. VISTA is an immune checkpoint molecule for human T cells. *Cancer Res* (2014) **74**(7):1924–32. doi:10.1158/0008-5472.CAN-13-1504
161. Flies DB, Han X, Higuchi T, Zheng L, Sun J, Ye JJ, et al. Coinhibitory receptor PD-1H preferentially suppresses CD4(+) T cell-mediated immunity. *J Clin Invest* (2014) **124**(5):1966–75. doi:10.1172/JCI74589
162. Liu J, Yuan Y, Chen W, Putra J, Suriawinata AA, Schenk AD, et al. Immune-checkpoint proteins VISTA and PD-1 nonredundantly regulate murine T-cell responses. *Proc Natl Acad Sci U S A* (2015) **112**(21):6682–7. doi:10.1073/pnas.1420370112
163. Wang L, Le Mercier I, Putra J, Chen W, Liu J, Schenk AD, et al. Disruption of the immune-checkpoint VISTA gene imparts a proinflammatory phenotype with predisposition to the development of autoimmunity. *Proc Natl Acad Sci U S A* (2014) **111**(41):14846–51. doi:10.1073/pnas.1407447111
164. Flies DB, Higuchi T, Chen L. Mechanistic assessment of PD-1H coinhibitory receptor-induced T cell tolerance to allogeneic antigens. *J Immunol* (2015) **194**(11):5294–304. doi:10.4049/jimmunol.1402648
165. Le Mercier I, Chen W, Lines JL, Day M, Li J, Sergent P, et al. VISTA regulates the development of protective antitumor immunity. *Cancer Res* (2014) **74**(7):1933–44. doi:10.1158/0008-5472.CAN-13-1506
166. Bharaj P, Chahar HS, Alozie OK, Rodarte L, Bansal A, Goepfert PA, et al. Characterization of programmed death-1 homologue-1 (PD-1H) expression

and function in normal and HIV infected individuals. *PLoS One* (2014) **9**(10):e109103. doi:10.1371/journal.pone.0109103

167. Chapman PB, D'Angelo SP, Wolchok JD. Rapid eradication of a bulky melanoma mass with one dose of immunotherapy. *N Engl J Med* (2015) **372**(21):2073–4. doi:10.1056/NEJMc1501894
168. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N Engl J Med* (2015) **373**(1):23–34. doi:10.1056/NEJMoa1504030
169. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med* (2013) **369**(2):122–33. doi:10.1056/NEJMoa1302369

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REVIEW
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Alarmin' immunologists: IL-33 as a putative target for modulating T cell-dependent responses

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IL-33 is a known member of the IL-1 cytokine superfamily classically named "atypical" due to its diverse functions. The receptor for this cytokine is the ST2 chain (or IL-1RL1), part of the IL-1R family, and the accessory chain IL-1R. ST2 can be found as both soluble and membrane-bound forms, property that explains, at least in part, its wide range of functions. IL-33 has increasingly gained our attention as a potential target to modulate immune responses. At the beginning, it was known as one of the participants during the development of allergic states and other Th2-mediated responses and it is now accepted that IL-33 contributes to Th1-driven pathologies as demonstrated in animal models of experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis, and trinitrobenzene sulfonic acid-induced experimental colitis, among others. Interestingly, current data are placing IL-33 as a novel regulator of immune tolerance by affecting regulatory T cells (Tregs); although the mechanism is not fully understood, it seems that dendritic cells and myeloid suppressor-derived cells may be cooperating in the generation and/or establishment of IL-33-mediated tolerance. Here, we review the most updated literature on IL-33, its role on T cell biology, and its impact in immune tolerance.

Keywords: IL-33, T cells, tolerance, transplantation

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Introduction

In 2005, IL-33 was first described as a member of the IL-1 family using computational sequence analysis, which revealed the existence of a β-trefoil-fold structure in its C-terminal domain, a characteristic feature of IL-1 family members such as IL-1β and IL-18 (1). In the same work, it was also found that IL-33 interacts with the IL-1 family receptor ST2, previously described as an orphan receptor expressed on Th2 cells and mast cells. When activated with IL-33, ST2 promotes Th2 cell responses over Th1 responses (1), and negatively regulates TLR–IL-1R receptor signaling (2).

IL-33 is known as an *alarmin* because of its high expression in endothelial and epithelial cells exposed to tissue damage or pathogen encounter, in addition to its accumulation in the nucleus of those cells, similar to other alarmin members of the IL-1 family such as HMGB1 and IL-1α (3). Therefore, upon injury or infection, IL-33 is released from the nucleus of endothelial cells to the extracellular space where it can signal and activate immune cells, such as mast cells, eosinophils, basophils, natural killer cells, and T cells (as many other functions described in this review) (**Figure 1**) (4).

Human IL-33 is a 30 kDa protein that shares 54% amino acid identity with its mouse homolog (5). Along its 270 amino acid sequence, we can describe a distinctive C-terminal IL-1-like cytokine domain of the IL-1 cytokine family and a central domain (1). IL-33 lacks a clear signal peptide, but it is synthesized with an N-terminal pro-peptide containing a nuclear localization domain (3, 6), which seems to be essential to IL-33 function *in vivo* because specific deletion of this nuclear domain (using a *knock-in* mouse model) results in a systemic, ST2-dependent, non-resolving lethal inflammation due to constitutive release of IL-33 into the circulation (7).

FIGURE 1 | IL-33 diversity of functions.

As other members of the IL-1 family, IL-33 can suffer post-translational modifications resulting in two different forms: the full-length protein (proIL-33) and the processed or "mature" form (mIL-33). mIL-33 is formed after proIL-33 cleavage in its N-terminal domain, but the cleavage sequence is different compared to other members of the IL-1 family, such as proIL-1β and IL-18 (8) (**Figure 2**). In the extracellular space, IL-33 binds to its membrane receptor ST2 activating the MyD88-dependent pathway, which is involved in cytokine secretion, cell activation, and differentiation (9). IL-33 can also bind to the soluble form (sST2) of its receptor, which corresponds to a decoy receptor that captures IL-33, avoiding or blocking intracellular signaling (10). Recently, it has been highlighted that the two forms of IL-33 (proIL-33 and mIL-33) are biologically active and they can trigger different immune responses (11).

The nuclear localization of IL-33 led to study a possible role in controlling gene expression, as discussed below.

IL-33 as a Transcription Factor

As mentioned, IL-33 sequence does not contain a clear signal peptide, suggesting an (ancestral) intracellular localization. This observation suggests that IL-33 has a role as intracellular factor. Based on the similarities of IL-33 with other members of the IL-1 cytokine family, and the high-mobility group box-1 (HMGB1) protein, Carriere and colleagues studied the sequence, molecular, and cellular function

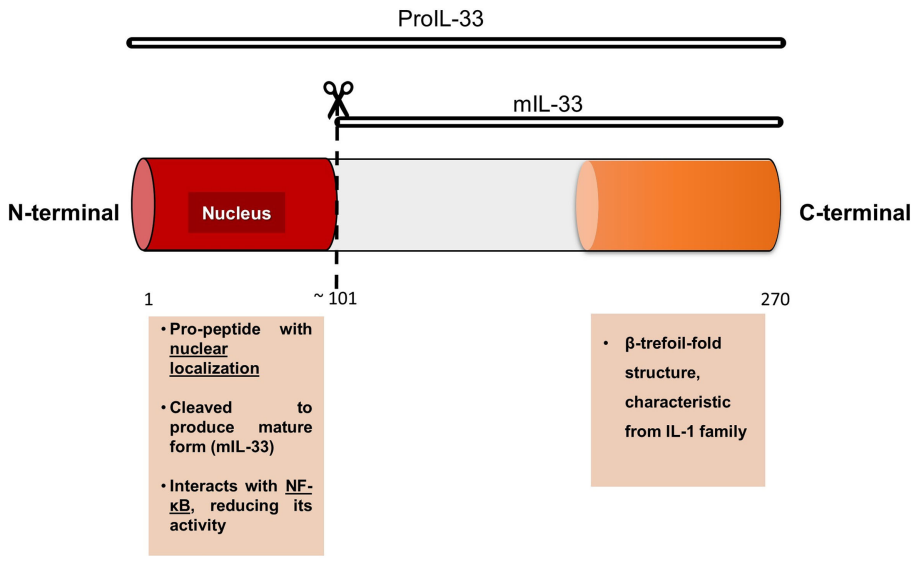

FIGURE 2 | Model of IL-33 protein, with its two principal domains and functions.

of IL-33. In the first set of experiments, the group confirmed IL-33 nuclear localization, and interestingly, this location overlapped with high concentrations of DNA, suggesting that endogenous IL-33 binds to heterochromatin. This observation was confirmed using GFP-tagged IL-33 constructs, which, in addition, indicated that IL-33 binds to mitotic chromatin (12). Sequence and mutagenic experiments indicated that the N-terminal section of the protein is necessary for nuclear targeting, binding to heterochromatin and mitotic chromosomes, specifically the homodomain-like helix–turn–helix segment. Making the association that heterochromatin relates with unreachable or unexposed DNA, the investigators assayed IL-33 gene expression regulatory properties using a luciferase-based reporter system and showed that IL-33 acts as a repressor factor (12). In another report, it was described that endogenous IL-33 interacts physically with the transcription factor NF-κB. By performing co-immunoprecipitation experiments and microscopy, the investigators demonstrated that IL-33 binds to a free and activated NF-κB. In other words, IL-33 does not interact with κB (13). Supporting Carriere's report, this group also found that IL-33 N-terminal sequence interacts with NF-κB, reducing its binding to DNA and repressing NF-κB transcription factor activity, as shown in EMSA and luciferase-based reporter experiments. Altogether, these reports indicate that endogenous IL-33 acts as a transcription factor (**Figure 1**).

A Multifaceted Cytokine

IL-33 and APCs

In addition to the role in the activation of the immune responses, IL-33 has also different roles in T cell polarization by modulating APCs. For example, IL-33 promotes the expansion of APCs, principally dendritic cells (DCs), by stimulating the secretion of basophils-derived GM-CSF (14). In a different study, Rank and colleagues found that DCs express high levels of intracellular ST2, and that the *in vitro* treatment of DCs with IL-33 induces the secretion of IL-6, upregulates the expression levels of MHC-II and CD86, and triggers the polarization of naïve T cells toward a Th2 phenotype (15). Similarly, Besnard and colleagues showed that IL-33 exposure induces the recruitment and activation of DCs in the lung, and their posterior migration to lymph nodes for antigen presentation in an allergic airway inflammation model (16). Alternatively, an indirect role of IL-33 on DCs was described by Duan and colleagues, who showed that IL-33 indirectly modulates intestinal DCs phenotype toward a tolerogenic profile given by the expression of CD103+CD11c+ (17). Supporting this potential tolerogenic effect, it was recently shown that IL-33 promotes IL-2 secretion by DCs, favoring the expansion of Foxp3+ Tregs cells (18).

IL-33 and CD4+ T Cells

A lot has been described about the abilities of IL-33 to modulate the immune response. One of our particular interests is the capacity to direct CD4+ T cells polarization to different phenotypes, with the purpose to manipulate immunity under pathogenic conditions, favoring a healthy state. In this line, we believe IL-33 is a good candidate to exploit, and here we review IL-33 effects on different T cells subsets (**Table 1**).

IL-33 on Th1/Th17

The context in which IL-33 is present influences directly the kind of immune response triggered. Thus, in certain cases, IL-33 can induce a Th1- or Th17-type response (**Figure 1**). These outcomes participate in the development and establishment of inflammatory responses that usually relate to diseases, such as autoimmunity, or to detrimental inflammatory responses such as graft-versus-host disease (GvHD) for the case of transplantation. Because of this, to understand the conditions when this cytokine would induce an inflammatory response over a tolerogenic one, it is essential when considering candidates for therapeutic interventions.

We believe that IL-33 versatility has contributed to the contradictory studies using animal models. For example, in the experimental autoimmune encephalomyelitis (EAE) model, some investigators have reported that IL-33 blockade during the developing stage of MOG-induced EAE reduces the severity of the disease, in part, due to the inhibition of MOG-dependent IL-17 and IFN-γ production. Unexpectedly, the treatment with exogenous IL-33 increased the severity of the disease by enhancing the production of these cytokines. Conversely, the treatment with an anti-IL-33 antibody reversed the symptoms and improved the state of the mice (19). In contrast with these findings, Jiang and colleagues observed an improvement in EAE mice after IL-33 treatment (20). When comparing both works, we identified differences that may contribute to obtain such contradictory results as the immunization (MOG peptide) site/location, timing, and peptide amount. Nevertheless, the kinetics and severity of the disease are equal in both cases. In addition, IL-33 treatment differs in both works; Li et al. injects the mice every other day, since day 0–18 with 50 μg/kg; while Jiang's group treat the mice with 1 μg total from day 12 to 20 after immunization. These differences, especially in the treatment, can be related to the different results obtained, leading us to think that the effect given by IL-33 is time dependent and may affect different cell populations in each case. The IL-33 receptor ST2 can be expressed on astrocytes in the nervous system (37); thus, a potential impact of this cytokine on glial cells is very likely and may vary with the inflammatory state of them.

Similar to the EAE model, conflicting data are also reported for rheumatoid arthritis (RA). For example, some studies indicate that IL-33 is prejudicial for this disease because it induces a Th17-type response, which aggravates the symptoms (21, 22). In collagen-induced arthritis (CIA), it has been described that IL-33 has similar effects, developing a strong autoimmune response mediated by Th17 cells (22). According to these data, the blockade of ST2 helps to diminish the severity of CIA through a decrease in the production of IFN-γ and IL-17 (21, 23). However, other authors state that the role of IL-33 in this specific disease is not relevant. For example, Talabot-Ayer and colleagues showed in models of CIA and antigen-induced arthritis (AIA) that IL-33 knock-out mice develop the disease despite the lack of IL-33. They conclude that IL-33 does not play an essential role for the development of the disease, but it may contribute to the inflammatory environment (24).

Other disease linked to IL-33 is asthma. When IL-33 expression is deregulated, Th2 cells, mast cells, basophils, and others cells get

TABLE 1 | IL-33 effects in T cells.

Cell type	Experimental model	Observations	Reference
CD4 Thl/Thl7	EAE	IL-33 blockade during MOG-dependent development reduces disease severity by decreasing IFN- γ and IL-17	(19)
		Treatment with exogenous IL-33 increases disease severity through IFN-γ and IL-17 secretion αIL-33 treatment improves symptoms	
	EAE	IL-33 treatment improves symptoms	(20)
	RA, CIA	IL-33 induces Th1 and Th17 responses	(21–23)
		ST2 blockade reduces severity by diminishing IFN-γ and IL-17 production	
	CIA, AIA	Presence of IL-33 did not affect development of the disease	(24)
	OVA-induced allergic asthma	IL-33 induces the secretion of IL-6 and IL-1β from mast cells, promoting Th17 differentiation	(25)
CD4 Th2	Experimental atherosclerosis Innate immunity	IL-33 upregulates serum levels of Th2 cytokines and enriches for CD4+ST2+ T cells	(26)
		IL-33 acts on mast cells, basophils, eosinophils, type 2 innate lymphoid cells (ILC2), which promotes the secretion of Th2 cytokines, favoring Th2 polarization	(22, 27–29)
CD4 Tregs	Chronic colitis	Treatment with IL-33 reduces disease symptoms through a decrease on IFN-γ production, switching from a Th1 to a Th2 type response	(17, 30, 31)
		IL-33 affects directly Tregs in mice	
		IL-33 expands Tregs Foxp3+ST2+	
	EAE	IL-33-treated mice show an increase in Tregs and M2 macrophages	(20)
	Atherosclerosis	IL-33 prevents the development of atherosclerosis in $Apo3^{-/-}$ mice	(26, 32)
		IL-33 treatment produces an increment in IL-4, IL-5, and IL-13 production and a decrease in IFN-γ levels	
		IL-33/ST2 axis is involved in the development of the disease	
	Transplantation	Heart-transplanted mice treated with IL-33 show increased graft survival	(33)
		IL-33 treatment increases the number of CDllb + GRlint MDSC and Tregs, in addition to a less IL-17 production and elevated levels of IL-5, IL-13, and IL-10	
CD8		IL-33 is up-regulated in CD8+ T cells	(34)
		ST2 expression is T-bet-dependent	
		IL-33 plus IL-12 enhance CD8+ T cell effector phenotype	
	Lymphocytic choriomeningitis virus	IFN-γ, ST2, and IL-33 expressions were upregulated after infection	(35)
	Herpesvirus	IL-33 signaling is necessary for an effective CTL response	(35)
	Tumor	Tumor-derived IL-33 inhibits tumor growth and blocks the entrance of tumor-infiltrating lymphocytes (TILs)	(36)
		Mice-bearing IL-33-secreting tumor have more activated and differentiated CD8+ T cells, which is related to T-bet and Eomes expression	

activated triggering the expression of cytokines and chemokines that characterizes the disease (38). However, in a model of OVA-induced allergic asthma, the authors described a predominant Th17-type response, which is induced by the activation of ST2/IL-33 signaling on mast cells. The authors observed that mast cells from IL-33-treated mice produced IL-1β and IL-6, collaborating with Th17 cells development (25).

These few examples show us the pleiotropic abilities of this cytokine, which can direct CD4+ T cells differentiation to Th1 and Th17, causing and participating in different inflammatory states and diseases.

IL-33 on Th2

As mentioned earlier, IL-33 can also modulate Th2 type responses (**Figure 1**) in addition to its effect on Th1 cells. *In vitro* and *in vivo* data suggest that IL-33 indirectly promotes Th2 responses functioning as a chemoattractant for this type of cells (39, 40). Other studies demonstrated that IL-33 could directly act on CD4+ST2+ Th2 cells promoting the secretion of IL-4, IL-5, and IL-13 (40–42). IL-33 has shown a protective role in the $ApoE^{-/-}$ experimental atherosclerosis mice model, increasing serum levels of Th2-type cytokines, and enriching ST2+CD4+ T cells in lymph nodes (26). These observations indicate that IL-33 affects Th2 cell cytokine production in a TCR-independent fashion. Moreover, IL-33 promotes Th2-type responses by signaling on other immune cells rather than CD4+ T cells. In this case, it has been reported that IL-33 induces mast cells activation, maturation, and cytokine production such as IL-5, IL-6, IL-8, and IL-13 (22, 27, 43). In addition to this, IL-33 can also modulate Th2 immunity by acting on basophils, eosinophils, and more recently described, on newly identified type-2 innate lymphoid cells (ILC2), which includes nuocytes, natural helper cells, and

innate helper type-2 cells. On these cells, IL-33 promotes IL-5 and IL-13 secretion (29, 42, 43–45).

Taking all together, there are sufficient indications supporting the role of IL-33, either directly or indirectly, in Th2-mediated immune responses.

IL-33 on Tregs

In addition to the evidence mentioned above, it is reported that IL-33 can also expand and promote Tregs accumulation, among other effects, helping to establish tolerance (**Figure 1**). Different animal models support this evidence, from autoimmunity to organ transplantation settings.

For the case of autoimmunity, one of the models studied is chronic colitis. In this approach, the investigators observed that mice treated with exogenous IL-33 manifested a decrease in disease symptoms, which was associated to a down-regulation on IFN-γ production. This intervention on the cytokine environment led to a shift of the T helper response, from Th1 to Th2, reducing inflammation in the intestine (30). This work demonstrated the capacity of IL-33 to modulate immune responses in this organ, but it does not imply necessarily a direct role for Tregs. However, in another model of colitis, which is induced by trinitrobenzene sulfonic acid (TNBS), the protective role of IL-33 was also observed. In this case, the authors evaluated the contribution of Tregs by depleting these cells in IL-33-treated diseased animals, obtaining a loss in protection compared to non-depleted animals or, in other words, Tregs contribute to IL-33-mediated protection (17). One could argue that IL-33 may or may not be acting on CD4+ T cells because ST2 is expressed by a wide range of cells; nevertheless, a Treg-dependent response is seen demonstrating that CD4+ T cells can rapidly react (directly or not) to IL-33 stimulation. Supporting these findings, Schiering and colleagues described that IL-33 treatment expands Tregs, increasing Foxp3 and ST2 expression in the spleen of mice with ongoing colitis. In this report, IL-33 treatment did not disturb effector T cell function, but adoptive transfer experiments indicated that IL-33 acts on thymic Tregs (natural Tregs, nTregs) rendering a stable phenotype by "fixing" Foxp3 expression (therefore better suppressors) (31).

Since the expression of ST2 is widely distributed in cells of the nervous system, the role of IL-33 signaling has been also studied in the EAE model (46). In this experimental setting, IL-33 treatment incremented the frequency of Tregs and polarized macrophage phenotype toward M2 type in the spinal cord of EAE mice (20).

Furthermore, in atherosclerosis, a typical Th1-type pathology in which the frequency and function of Tregs are reduced, it was observed that IL-33 treatment of Apo3$^{-/-}$ mice with a high-fat diet (model for this disease) prevented the development of atherosclerotic plaques, which is the first symptom of this disease. In addition, the authors obtained an increment on IL-4, IL-5, and IL-13 production, leading to a decrease in IFN-γ production, resulting in a overall switch from Th1 to a protective Th2-type response (26). A couple of years later, Wasserman and colleagues observed, in the same model, that mice under IL-33 treatment were unable to increase the number of cells with regulatory properties as wild-type controls (32). They also observed a poor expression of ST2 on CD4+ T cells from sick mice, which may be a reason for the unresponsiveness to exogenous IL-33 (and the little increase of Tregs under this treatment). However, the lack of membrane-bound ST2 is explained by a mayor concentration of soluble ST2 (quantified by ELISA), which is a decoy receptor that avoids the downstream signaling when IL-33 is bound, preventing response to the cytokine (32). Altogether, this evidence suggests that the IL-33/ST2 axis is involved in the development of atherosclerosis, and proposes that a problem on IL-33 signaling may affect the ability of the organism to respond adequately to an unbalance of the organism homeostasis.

With respect to the transplantation field, in 2011, Brunner and collaborators showed that periodic IL-33 treatment of heart-transplanted mice resulted in an extended graft survival (33). This observation was associated to an increment in CD4+Foxp3+ Tregs population, which modulates the response to the graft. Also, the CD11b + Gr1int myeloid suppressor-derived cells (MDSCs) were augmented, contributing to the acceptance of the graft. Another interesting point made in this study is the complete change in the cytokine pattern: mice treated with IL-33 showed a decreased of IL-17A production, but an increase of IL-13, IL-5, and IL-10 production of *in vitro*-cultured, purified graft-infiltrating cells (33). With these data, the authors associated the improvement on graft survival with a Th2-type response, which favors the induction of regulatory cell populations such as Tregs and MDSCs.

In this same topic, our own group has seen an increment in CD4+FoxP3+ T cells in mice grafted with semi-allogeneic skin transplant and treated with IL-33, which is also related to a better state of the graft, a blockade in IFN-γ and IL-17 production and increased skin graft survival.

The information above shows that IL-33 participates in the induction of tolerance by targeting CD4+ T cells and some APC populations, orchestrating an immune response to favor an immunoregulatory status of the organism.

The data from all models (autoimmunity and transplantation) indicate that IL-33 is involved or may act at different stages of the immune response resulting (most likely) in a protective immunity for the organism.

IL-33 and CD8+ T Cells

The first role of IL-33 in immunity was linked to the innate-type response. But later, several reports showed that IL-33 is able to modulate adaptive immunity, mainly by acting directly on CD4+ T cells. Until 2009, the role of IL-33 on cytotoxic CD8+ T cells (Tc cells) was totally unknown. The first study elucidating a potential function of IL-33 on this T cell population was reported by Yang and collaborators. His work focused on the contribution of IL-33, in a TCR-dependent and -independent fashion, on human and murine CD8+ T cell biology *in vitro*. First, they performed gene arrays analysis on murine type-I Tc cells and found that IL-33 was one of the genes highly upregulated (34). Since IL-33 affects Th1, Th2, and Th17 differentially, the investigators stimulated Tc cells under type-1, -2, and -17 conditions, and measured ST2 mRNA. The results indicated that ST2 is up-regulated on Tc1 and Tc17 cells, but IL-4 inhibited ST2 mRNA on Tc2 cells. Interestingly, ST2 expression on Tc1 seems to be dependent on T-bet, as shown in experiments using *T-bet* KO CD8+ T cells. Conversely, exogenous IFN-γ did not alter ST2 expression neither at mRNA nor at the protein level (34). Considering that ST2 expression depends on

TCR stimulation and T-bet, the authors evaluated the contribution of exogenous IL-33 on CD8+ T cell biology. According to the data reported above, IL-33 plus IL-12 upregulated IFN-γ, T-bet, and Blimp-1 expression on Tc1 cells, downregulating Eomes and IL-7R levels (T cell memory-associated molecules), without affecting the production of Granzymes or Perforins. Taking together, IL-33 and IL-12 enhance effector phenotype on CD8+ T cells (34). As in Th1 cells, these results are partially dependent on Gadd45b, a protein involved in CD4+ T cell arrest and differentiation (34). Another interesting report focused on identifying molecules involved in the induction of anti-viral responses. In this work, mice were infected with lymphocytic choriomeningitis virus (LCMV), and their splenocytes were harvested to analyze gene expression changes triggered by LCMV infection. From a panel of cytokines and other molecules, IFN-γ and IL-33 were the most up-regulated ones, in addition to ST2 (35). IL-33 contribution to anti-LCMV response was evaluated infecting *Il-33*$^{-/-}$, *Il1rl1-Fc*, and *Il1rl1*$^{-/-}$mice, resulting in an impairment of the cytotoxic T lymphocyte (CTL)-mediated response. In a similar approach, ST2-deficient animals infected with murine herpes virus 68 (MHV-68) presented elevated viral titers, which it was reversed when administering exogenous IL-33, suggesting that IL-33 signaling is necessary for virus clearance or an effective CTL response. This hypothesis was finally proved performing bone marrow chimera experiments, in which only wild-type CTLs were responsive to LCMV infection versus the ST2-deficient counterpart, given in part by a reduced survival and the lack of granzyme B and CD107a expression (important molecules involved in cytotoxicity) by the deficient cells (35). On the other hand, this experimental approach permitted to identify the non-hematopoietic compartment as the main source of IL-33 during viral infection. Finally, a very recent manuscript describes the role of IL-33 on tumor immunology. In this report, the investigators engineered two tumor cell lines to over-express (and secrete) IL-33. When injected into mice, the IL-33-producing tumor grew less, and the tumor infiltrating lymphocytes (TILs) were more abundant than in mice injected with control tumor cells. Moreover, when TILs were characterized, CD8+ T cells were enriched as in controls, but they displayed a more activated and differentiated phenotype given by elevated expression of the transcription factors T-bet and Eomes, higher IFN-γ secretion, and granzyme B production, demonstrating that IL-33 controls CD8+ T cells differentiation and effector function, which can be manipulated for anti-tumor therapy (36).

Taken together, the findings described above indicate that not only CD4+ T cells get triggered by IL-33 but also CD8+ T cells, supporting the idea that IL-33 may modulate T cell-dependent functions, which in turn can be reflected on the nature of the adaptive immune response.

Future Perspectives

The information above clearly manifests the variety of roles that IL-33 exerts in the immune system, considering both innate and adaptive types of responses. To manipulate and translate IL-33 functions to treat disease and restore immune homeostasis or tolerance, we need to understand in a more detailed way the IL-33 participation during autoimmunity and graft rejection. Current studies using animal models show that IL-33 is present in the inflammatory response triggered during disease, but extensive studies are required using cells and tissue from patients. Recapitulating the roles of IL-33 in human settings can open new opportunities to design immunological therapies. Of our interest is the field of Tregs, where IL-33 positively impacts their biology, by mainly expanding or increasing their number *in vivo*. Is this response also observed on human Tregs? Are patient's Tregs sensitive to IL-33 treatment? Is ST2 expression altered during disease? Are IL-33-expanded Tregs able to migrate to inflamed tissue? Many questions are still unsolved.

At present, there are some biological therapies that target cytokines for treating diseases; thus, a similar approach can be used for IL-33. Similar to IL-33, IL-2 therapy was sought to enrich for Tregs, since this cytokine acts as a growth factor for T cells. In an animal model of diabetes, low dose of IL-2 promoted Treg accumulation and function, and favored a switch in cytokine milieu given by a reduction on IFN-γ production. Interestingly, this low dose of IL-2 did not alter the frequency of other cell types (including effector T cells), but Treg number (47). In humans, IL-2 administration has also showed benefits, such as an increase in functional Treg numbers and a reduction in inflammatory signs [GVHD and vasculitis patients (48, 49)]. Alternatively, an antibody anti-TNFα was designed to treat RA. This cytokine is normally produced in response to local injury; however, it drives an exacerbated inflammation producing tissue damage when secreted in excess. In RA, TNF-α is present in the synovial fluid from patients. At date, there are five biological inhibitors approved by the FDA, three of them are monoclonal antibodies (infliximab, adalimumab, and golimumab); Etanercept is a fusion protein of two TNFR2 receptor extracellular domains plus the Fc fragment of human immunoglobulin 1, and Certolizumab is a humanized Fab fragment conjugated to polyethylene glycol (50). Although the patient life quality improves, they are not an effective treatment due to a loss of effectiveness or severe secondary effects, such as higher risk to serious infections or lymphoma (50). Other treatments targeting cytokines or their receptors include the anti-IL-6R antibody. This approach was developed considering the high amounts of IL-6 in the synovial fluid of patients. The receptor for this cytokine, IL-6Rα is found in a wide range of cells, including T cells, monocytes, activated B cells, neutrophils, and hepatocytes (51). Recently, a monoclonal treatment has been proved (Tocilizumab), which is capable to block IL-6R, producing an improvement in the symptoms. The main problems with this therapy are the secondary effects associated with its use. Since IL-6 is a very pleiotropic cytokine, many tissues and/or organs get affected, including elevated propensity to infections, malignant tumors, changes in the blood and lipid profile, hepatic problems, and cardiovascular danger (52).

Until date, specificity is the main obstacle for the application of this type of approaches, considering that (all) cells expressing or producing the cytokine and its receptor could get triggered by the administered drug. Therefore, when thinking in the possibility to use IL-33 for human treatment, we need to be specific regarding its target, because of its wide effects on cell types and, consequently, in the microenvironment. This phenomenon is related to the signaling pathways triggered during the encounter between the cytokine

and its receptor. For this reason, a better knowledge on IL-33 signaling and its interaction with others molecules are really necessary. Based in the different responses observed in a same animal model for diseases, we could think that dosage, timing, microenvironment, and state of the disease are key variables that may affect the final outcome of IL-33 treatment; thus, determining when these factors are aligned to favor a polarization to Tregs (for autoimmunity or block graft rejection) would be useful to act positively in the patient.

Lastly, the administration method is not less important. For delivery of sensitive molecules, such as cytokines, an interesting solution is the use of novel technologies. Nanomedicine supports the control, repair, and improvement of the human biological system working from the molecular level, and it has been proposed as a possible solution for the controlled release of a desired molecule. It consists in establishing nanostructures to carry a drug to an affected area, encapsulating, and releasing it at its destination under specific conditions (53). This dynamic mechanism is called nanotherapy and it is characterized by several benefits including improvement in disease detection and good rate of absorption and treatment of patients. Among the nanostructures designed, we can find nanoparticles, nanocapsules, dendrimers, liposomes, micelles, nanotubes, and microgeles, each of one with characteristic properties. Applying the above to IL-33 may be a great opportunity to create a therapy through specific targeting leading to an effective cure to restore immune tolerance (54).

In summary, there is still a lot to know and understand about IL-33; however, the results shown to date gives us positive expectations on a new therapy involving this cytokine.

References

1. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* (2005) **23**:479–90. doi:10.1016/j.immuni.2005.09.015
2. Brint EK, Xu D, Liu H, Dunne A, McKenzie AN, O'Neill LA, et al. ST2 is an inhibitor of interleukin 1 receptor and toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat Immunol* (2004) **5**:373–9. doi:10.1038/ni1050
3. Moussion C, Ortega N, Girard JP. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? *PLoS One* (2008) **3**:e3331. doi:10.1371/journal.pone.0003331
4. Cayrol C, Girard JP. The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. *Proc Natl Acad Sci U S A* (2009) **106**:9021–6. doi:10.1073/pnas.0812690106
5. Palmer G, Gabay C. Interleukin-33 biology with potential insights into human diseases. *Nat Rev Rheumatol* (2011) **7**:321–9. doi:10.1038/nrrheum.2011.53
6. Liu X, Hammel M, He Y, Tainer JA, Jeng U-S, Zhang L, et al. Structural insights into the interaction of IL-33 with its receptors. *Proc Natl Acad Sci U S A* (2013) **110**(37):14918–23. doi:10.1073/pnas.1308651110
7. Bessa J, Meyer CA, de Vera Mudry MC, Schlicht S, Smith SH, Iglesias A, et al. Altered subcellular localization of IL-33 leads to non-resolving lethal inflammation. *J Autoimmun* (2014) **55**:33–41. doi:10.1016/j.jaut.2014.02.012
8. Lefrancais E, Cayrol C. Mechanisms of IL-33 processing and secretion: differences and similarities between IL-1 family members. *Eur Cytokine Netw* (2012) **23**:120–7. doi:10.1684/ecn.2012.0320
9. Kakkar R, Lee RT. The IL-33/ST2 pathway: therapeutic target and novel biomarker. *Nat Rev Drug Discov* (2008) **7**:827–40. doi:10.1038/nrd2660
10. Hayakawa H, Hayakawa M, Kume A, Tominaga S. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. *J Biol Chem* (2007) **282**:26369–80. doi:10.1074/jbc.M704916200
11. Talabot-Ayer D, Lamacchia C, Gabay C, Palmer G. Interleukin-33 is biologically active independently of caspase-1 cleavage. *J Biol Chem* (2009) **284**:19420–6. doi:10.1074/jbc.M901744200
12. Carriere V, Roussel L, Ortega N, Lacorre DA, Americh L, Aguilar L, et al. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc Natl Acad Sci U S A* (2007) **104**(1):282–7. doi:10.1073/pnas.0606854104
13. Ali S, Mohs A, Thomas M, Klare J, Ross R, Schmitz ML, et al. The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription. *J Immunol* (2011) **187**:1609–16. doi:10.4049/jimmunol.1003080
14. Mayuzumi N, Matsushima H, Takashima A. IL-33 promotes DC development in BM culture by triggering GM-CSF production. *Eur J Immunol* (2009) **39**:3331–42. doi:10.1002/eji.200939472
15. Rank MA, Kobayashi T, Kozaki H, Bartemes KR, Squillace DL, Kita H. IL-33-activated dendritic cells induce an atypical TH2-type response. *J Allergy Clin Immunol* (2009) **123**:1047–54. doi:10.1016/j.jaci.2009.02.026
16. Besnard AG, Togbe D, Guillou N, Erard F, Quesniaux V, Ryffel B. IL-33-activated dendritic cells are critical for allergic airway inflammation. *Eur J Immunol* (2011) **41**:1675–86. doi:10.1002/eji.201041033
17. Duan L, Chen J, Zhang H, Yang H, Zhu P, Xiong A, et al. Interleukin-33 ameliorates experimental colitis through promoting Th2/Foxp3(+) regulatory T-cell responses in mice. *Mol Med* (2012) **18**:753–61. doi:10.2119/molmed.2011.00428
18. Matta BM, Lott JM, Mathews LR, Liu Q, Rosborough BR, Blazar BR, et al. IL-33 Is an unconventional Alarmin that stimulates IL-2 secretion by dendritic cells to selectively expand IL-33R/ST2+ regulatory T cells. *J Immunol* (2014) **193**:4010–20. doi:10.4049/jimmunol.1400481
19. Li M, Li Y, Liu X, Gao X, Wang Y. IL-33 blockade suppresses the development of experimental autoimmune encephalomyelitis in C57BL/6 mice. *J Neuroimmunol* (2012) **247**:25–31. doi:10.1016/j.jneuroim.2012.03.016
20. Jiang HR, Milovanovic M, Allan D, Niedbala W, Besnard AG, Fukada SY, et al. IL-33 attenuates EAE by suppressing IL-17 and IFN-gamma production and inducing alternatively activated macrophages. *Eur J Immunol* (2012) **42**:1804–14. doi:10.1002/eji.201141947
21. Palmer G, Talabot-Ayer D, Lamacchia C, Toy D, Seemayer CA, Viatte S, et al. Inhibition of interleukin-33 signaling attenuates the severity of experimental arthritis. *Arthritis Rheum* (2009) **60**:738–49. doi:10.1002/art.24305
22. Xu D, Jiang HR, Kewin P, Li Y, Mu R, Fraser AR, et al. IL-33 exacerbates antigen-induced arthritis by activating mast cells. *Proc Natl Acad Sci U S A* (2008) **105**:10913–8. doi:10.1073/pnas.0801898105
23. Leung BP, Xu D, Culshaw S I, McInnes B, Liew FY. A novel therapy of murine collagen-induced arthritis with soluble T1/ST2. *J Immunol* (2004) **173**:145–50. doi:10.4049/jimmunol.173.1.145
24. Talabot-Ayer D, Martin P, Seemayer CA, Vigne S, Lamacchia C, Finckh A, et al. Immune-mediated experimental arthritis in IL-33 deficient mice. *Cytokine* (2014) **69**:68–74. doi:10.1016/j.cyto.2014.05.007
25. Cho KA, Suh JW, Sohn JH, Park JW, Lee H, Kang JL, et al. IL-33 induces Th17-mediated airway inflammation via mast cells in ovalbumin-challenged mice. *Am J Physiol Lung Cell Mol Physiol* (2012) **302**:L429–40. doi:10.1152/ajplung.00252.2011
26. Miller AM, Xu D, Asquith DL, Denby L, Li Y, Sattar N, et al. IL-33 reduces the development of atherosclerosis. *J Exp Med* (2008) **205**(2):339–46. doi:10.1084/jem.20071868
27. Ho LH, Ohno T, Oboki K, Kajiwara N, Suto H, Iikura M, et al. IL-33 induces IL-13 production by mouse mast cells independently of IgE-FcepsilonRI signals. *J Leukoc Biol* (2007) **82**:1481–90. doi:10.1189/jlb.0407200
28. Junttila IS, Watson C, Kummola L, Chen X, Hu-Li J, Guo L, et al. Efficient cytokine-induced IL-13 production by mast cells requires both IL-33 and IL-3. *J Allergy Clin Immunol* (2013) **132**:704–12. doi:10.1016/j.jaci.2013.03.033
29. Barlow JL, Peel S, Fox J, Panova V, Hardman CS, Camelo A, et al. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. *J Allergy Clin Immunol* (2013) **132**:933–41. doi:10.1016/j.jaci.2013.05.012

30. Grobeta P, Doser K, Falk W, Obermeier F, Hofmann C. IL-33 attenuates development and perpetuation of chronic intestinal inflammation. *Inflamm Bowel Dis* (2012) **18**:1900–9. doi:10.1002/ibd.22900
31. Schiering C, Krausgruber T, Chomka A, Frohlich A, Adelmann K, Wohlfert EA, et al. The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* (2014) **513**:564–8. doi:10.1038/nature13577
32. Wasserman A, Ben-Shoshan J, Entin-Meer M, Maysel-Auslender S, Guzner-Gur H, Keren G. Interleukin-33 augments Treg cell levels: a flaw mechanism in atherosclerosis. *Isr Med Assoc J* (2012) **14**(10):620–3.
33. Brunner SM, Schiechl G, Falk W, Schlitt HJ, Geissler EK, Fichtner-Feigl S. Interleukin-33 prolongs allograft survival during chronic cardiac rejection. *Transpl Int* (2011) **24**:1027–39. doi:10.1111/j.1432-2277.2011.01306.x
34. Yang Q, Li G, Zhu Y, Liu L, Chen E, Turnquist H, et al. IL-33 synergizes with TCR and IL-12 signaling to promote the effector function of CD8+ T cells. *Eur J Immunol* (2011) **41**:3351–60. doi:10.1002/eji.201141629
35. Bonilla WV, Frohlich A, Senn K, Kallert S, Fernandez M, Johnson S, et al. The alarmin interleukin-33 drives protective antiviral CD8(+) T cell responses. *Science* (2012) **335**:984–9. doi:10.1126/science.1215418
36. Gao X, Wang X, Yang Q, Zhao X, Wen W, Li G, et al. Tumoral expression of IL-33 inhibits tumor growth and modifies the tumor microenvironment through CD8+ T and NK cells. *J Immunol* (2015) **194**:438–45. doi:10.4049/jimmunol.1401344
37. Andre R, Lerouet D, Kimber I, Pinteaux E, Rothwell NJ. Regulation of expression of the novel IL-1 receptor family members in the mouse brain. *J Neurochem* (2005) **95**:324–30. doi:10.1111/j.1471-4159.2005.03364.x
38. Borish L, Steinke JW. Interleukin-33 in asthma: how big of a role does it play? *Curr Allergy Asthma Rep* (2011) **11**:7–11. doi:10.1007/s11882-010-0153-8
39. Kouzaki H, Iijima K, Kobayashi T, O'Grady SM, Kita H. The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. *J Immunol* (2011) **186**:4375–87. doi:10.4049/jimmunol.1003020
40. Louten J, Rankin AL, Li Y, Murphy EE, Beaumont M, Moon C, et al. Endogenous IL-33 enhances Th2 cytokine production and T-cell responses during allergic airway inflammation. *Int Immunol* (2011) **23**:307–15. doi:10.1093/intimm/dxr006
41. Kurowska-Stolarska M, Kewin P, Murphy G, Russo RC, Stolarski B, Garcia CC, et al. IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J Immunol* (2008) **181**:4780–90. doi:10.4049/jimmunol.181.11.8170
42. Pecaric-Petkovic T, Didichenko SA, Kaempfer S, Spiegl N, Dahinden CA. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* (2009) **113**(7):1526–34. doi:10.1182/blood-2008-05-157818
43. Imai Y, Yasuda K, Sakaguchi Y, Haneda T, Mizutani H, Yoshimoto T, et al. Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. *Proc Natl Acad Sci U S A* (2013) **110**(34):13921–6. doi:10.1073/pnas.1307321110
44. Nakae S, Morita H, Ohno T, Arae K, Matsumoto K, Saito H. Role of interleukin-33 in innate-type immune cells in allergy. *Allergol Int* (2013) **62**:13–20. doi:10.2332/allergolint.13-RAI-0538
45. Yasuda K, Muto T, Kawagoe T, Matsumoto M, Sasaki Y, Matsushita K, et al. Contribution of IL-33-activated type II innate lymphoid cells to pulmonary eosinophilia in intestinal nematode-infected mice. *Proc Natl Acad Sci U S A* (2012) **109**:3451–6. doi:10.1073/pnas.1201042109
46. McCarthy DP, Richards MH, Miller SD. Mouse models of multiple sclerosis: experimental autoimmune encephalomyelitis and Theiler's virus-induced demyelinating disease. *Methods Mol Biol* (2012) **900**:381–401. doi:10.1007/978-1-60761-720-4_19
47. Grinberg-Bleyer Y, Baeyens A, You S, Elhage R, Fourcade G, Gregoire S, et al. IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. *J Exp Med* (2010) **207**:1871–8. doi:10.1084/jem.20100209
48. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP III, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med* (2011) **365**:2055–66. doi:10.1056/NEJMoa1108188
49. Saadoun D, Rosenzwajg M, Joly F, Six A, Carrat F, Thibaul V, et al. Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. *N Engl J Med* (2011) **365**:2067–77. doi:10.1056/NEJMoa1105143
50. Thalayasingam N. Anti-TNF therapy. *Best Pract Res Clin Rheumatol* (2011) **25**:549–67. doi:10.1016/j.berh.2011.10.004
51. Hashizume M, Tan SL, Takano J, Ohsawa K, Hasada I, Hanasaki A, et al. Tocilizumab, A humanized anti-IL-6R antibody, as an emerging therapeutic option for rheumatoid arthritis: molecular and cellular mechanistic insights. *Int Rev Immunol* (2014) **34**(3):265–79. doi:10.3109/08830185.2014.938325
52. Smolen JS, Schoels MM, Nishimoto N, Breedveld FC, Burmester GR, Dougados M, et al. Consensus statement on blocking the effects of interleukin-6 and in particular by interleukin-6 receptor inhibition in rheumatoid arthritis and other inflammatory conditions. *Ann Rheum Dis* (2013) **72**(4):482–92. doi:10.1136/annrheumdis-2012-202469
53. Wagner V, Dullaart A, Bock A-K, Zweck A. The emerging nanomedicine landscape. *Nat Biotechnol* (2006) **24**:1211–7. doi:10.1038/nbt1006-1211
54. Liu Y, Miyoshi H, Nakamura M. Nanomedicine for drug delivery and imaging: A promising avenue for cancer therapy and diagnosis using targeted functional nanoparticules. *Int J Cancer* (2007) **120**:2527–37. doi:10.1002/ijc.22709

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REVIEW
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Role of dendritic cells in the induction of lymphocyte tolerance

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The ability of dendritic cells (DCs) to trigger tolerance or immunity is dictated by the context in which an antigen is encountered. A large body of evidence indicates that antigen presentation by steady-state DCs induces peripheral tolerance through mechanisms such as the secretion of soluble factors, the clonal deletion of autoreactive T cells, and feedback control of regulatory T cells. Moreover, recent understandings on the function of DC lineages and the advent of murine models of DC depletion have highlighted the contribution of DCs to lymphocyte tolerance. Importantly, these findings are now being applied to human research in the contexts of autoimmune diseases, allergies, and transplant rejection. Indeed, DC-based immunotherapy research has made important progress in the area of human health, particularly in regards to cancer. A better understanding of several DC-related aspects including the features of DC lineages, milieu composition, specific expression of surface molecules, the control of signaling responses, and the identification of competent stimuli able to trigger and sustain a tolerogenic outcome will contribute to the success of DC-based immunotherapy in the area of lymphocyte tolerance. This review will discuss the latest advances in the biology of DC subtypes related to the induction of regulatory T cells, in addition to presenting current *ex vivo* protocols for tolerogenic DC production. Particular attention will be given to the molecules and signals relevant for achieving an adequate tolerogenic response for the treatment of human pathologies.

Keywords: dendritic cells, DC-based immunotherapy, tolerance, Foxp3, regulatory T cells

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INTRODUCTION

Dendritic cells (DCs) are key controllers of innate and adaptive immunity and central regulators of immune tolerance (1). These functions are mainly due to the capacities of DCs to capture and process antigens and respond to pathogen- and self-derived danger signals in peripheral tissues. Additionally, migratory abilities allow competent DCs to mobilize into draining secondary lymphoid

Abbreviations: APCs, antigen-presenting cells; cDCs, conventional dendritic cells; DCs, dendritic cells; GILZ, glucocorticoid-induced leucine zipper; iDCs, inflammatory dendritic cells; IL, interleukin; iTregs, inducible regulatory T cells; MHC, major histocompatibility complex; pDCs, plasmacytoid dendritic cells; PD-L1, programed cell death ligand-1; PRRs, pattern recognition receptors; RA, retinoic acid; TGF-β, transforming growth factor beta; TLR, toll-like receptor; TolDCs, tolerogenic dendritic cells; Tregs, regulatory T cells.

organs where they engage, in the presence of specific cytokines, antigen-specific naïve $CD4^+$ and $CD8^+$ T cells, thus triggering the activation, proliferation, and migration of these cells to peripheral tissues (2, 3).

It has become increasingly clear in recent years that DCs are a heterogeneous population of leukocytes. Various distinct DC subtypes have been identified, including plasmacytoid DCs (pDCs), conventional DCs (cDCs), and inflammatory DCs (iDCs), the last of which display most of the archetypical features of DCs and originate from circulating monocytes during inflammation (4–7). The cDC subtype can be divided further, with mice presenting the $CD8\alpha^+/CD103^+$ and $CD11b^+$ subsets (8), and the human counterparts that express the markers BDCA-3 and BDCA-1, respectively (5). Even though DC subtypes share common features, such as activation in response to pathogen recognition, each DC population has specialized functions (4). For example, pDCs produce type I interferon for viral immunity (9, 10), and iDCs display antimicrobial functions by producing the tumor necrosis factor (TNF) and the inducible nitric oxide synthase during infections (5, 11). In the case of cDCs, these cells are highly efficient at coupling innate and adaptive immunity (8). Moreover, the $CD8\alpha^+/CD103^+$ cDC subset excels in the cross-presentation of antigens to cytotoxic $CD8^+$ T cells, while $CD11b^+$ cDCs efficiently prime naïve $CD4^+$ T cells (12).

Despite the identification of the DC lineages, it is currently unclear if there is a DC subtype with dedicated tolerogenic functions. Indeed, rather than a specialized population, evidence indicates that most DC subtypes can promote lymphocyte tolerance through T cell deletion, T cell anergy induction, and regulatory T cells (Tregs) induction (13). Furthermore, the tolerogenic features of DCs can be influenced by the microenvironment, expression of specific receptors that favor antigen presentation in subimmunogenic conditions, activation of specific intracellular signaling responses, and by pharmacological modulation, among other factors. A better understanding of these mechanisms will support the development of DC-based therapies that induce specific Tregs, which can be applied in the treatment of immune system pathologies that display over-activation against self-antigens as well as for the treatment of transplant rejection.

For the purpose of this review, we will provide an overview of the function of DCs in the establishment of lymphocyte tolerance, with focus on Tregs induction. We will also provide perspectives on the mechanisms that could be exploited to induce tolerogenic DCs (TolDCs) in the context of human immune diseases.

CONTRIBUTION OF DCs TO THE INDUCTION OF NATURAL AND PERIPHERAL Tregs

Dendritic cells have long been candidates for immunotherapy (14). In mice, these cells can be used to efficiently expand antigen-specific or polyclonal Tregs in autoimmune, transplant, and graft-versus-host diseases, among other immunological disorders (15–18). With the advent of intravital microscopy, the *in vivo* interactions between DCs and Tregs in lymphoid organs have been visualized (19). Related to this, some studies have found that endogenous Treg-DC interactions in the lymph node last longer than those established between DCs and conventional T cells in steady-state or during inflammation (20), thus highlighting the relevance of Treg and DC contacts in physiological conditions.

The establishment of central tolerance in the thymus predominantly depends on medullary thymic epithelial cells, which express the autoimmune regulator AIRE, a transcription factor required for the presentation of tissue-specific antigens to thymocytes (21, 22). In this context, the role of DCs in central tolerance appears limited, as demonstrated by studies showing that the thymic T cell compartment is unimpaired in mice displaying constitutive ablation of DCs (23, 24). However and despite these findings, it has been suggested that DCs could play a non-redundant role in the induction of natural Tregs (nTregs) (23, 25, 26). An advanced study of the T cell receptor repertoire in thymocytes showed that bone marrow-derived antigen-presenting cells (APCs), which include DCs, substantially contribute to the composition of the nTreg T cell receptor repertoire (26). Interestingly, $CD8\alpha^+$ cDCs can acquire and present autoimmune regulator-dependent antigens derived from medullary thymic epithelial cells to nTregs (26).

In addition to sustain nTreg selection, DCs can promote nTreg development in the thymus via the CD27–CD70 costimulation axis (27). Expression of CD70 on epithelial cells and DCs, particularly those of the $CD8\alpha^+$ subset, contributes to nTreg survival (27). Thus, there is growing evidence indicating that DCs could be actively involved in promoting thymic Treg development. Nevertheless, it remains to be discovered if the repertoire, frequency, and function of nTregs are dependent on a specialized DC subset.

In secondary lymphoid organs and in non-lymphoid tissues, the dynamics of DC and Treg populations are closely intertwined (28). This notion is derived from observations in $Foxp3^{DTR}$ mice, in which the depletion of Tregs leads to a notable increase in DCs frequency (29). The mechanism by which Tregs exert control over DC expansion is not fully understood, but it does depend on the cytokine Flt3L, a crucial regulator of DC commitment (8, 30). In fact, *in vivo* Flt3L treatment results in more DCs and a concomitant increase in the number of inducible Tregs (iTregs) (31). In contrast, $Flt3l^{-/-}$ mice show a 10-fold reduction in cDCs together with lower Treg cell counts (28). Even though evidence suggests that Flt3L leads to Treg expansion via a DC-dependent mechanism, this cytokine may also influence additional myeloid cell types, which in turn could impact Treg homeostasis independently of DCs. Nevertheless, data acquired from models of acute DC depletion, as triggered by administering a diphtheria toxin to CD11c-DTR mice, also demonstrated a two- or threefold reduction of iTregs (28, 32). These findings indicate that DCs are active regulators of Treg numbers *in vivo*.

Also in peripheral tissues, the maintenance of Tregs is dependent on costimulation by DCs. Despite some reports indicating that the absence of CD80/86 molecules on DCs leads to higher expression of Foxp3 (33), additional work has revealed that interactions mediated by CD80 and CD86 on APCs with the CD28 receptor on T cells are key for maintaining iTregs (34–37). These studies have been recently revisited in experiments using CD11c DTA

mice (38), which constitutively lack DCs (32). Findings demonstrate that the absence of CD80/86 in the CD11c$^+$ compartment results in a reduction of peripheral Tregs (32). Thus, in addition to major histocompatibility complex (MHC)-II molecules (28), iTreg maintenance critically relies on the expression of costimulatory molecules by DCs (32).

Another mechanism through which DCs can maintain Treg functions is via expression of the programed cell death ligand-1 (PD-L1) molecule. Interaction between PD-L1 on DCs and PD-1 receptor on T cells promotes the expression of Foxp3$^+$ Tregs, whereas blocking PD-L1 *in vivo* curtails Treg conversion during tumor challenge (39). Furthermore, the PDL1/2-PD1 interaction between DCs and Treg cells prevents autoimmunity of the central nervous system (40). In addition to the activation molecules, the migratory abilities of DCs may be relevant in promoting T cell tolerance. Indeed, migratory DCs have superior abilities than resident DCs to induce Treg development for the same antigen (41), which may be important to consider in the design of DC-based therapies.

Altogether, these findings indicate that DCs promote the induction of natural and iTregs via a variety of mechanisms that include the expression of multiple costimulatory and coinhibitory signaling pathways, the secretion of regulatory factors, migration, and the ability to present antigens. The advent of innovative models for selective ablation of cDCs using discriminating markers, such as the zinc finger transcription factor, zDC or the DC receptor, DNGR-1 (42, 43), will provide invaluable information toward addressing the role of DC lineages in the establishment of lymphocyte tolerance.

CONTRIBUTION OF DC SUBTYPES TO T CELL TOLERANCE

Current understanding dictates that antigen presentation in the absence of pathogen/danger detection leads to T cell tolerance of self or non-self antigens (4, 13). Nevertheless, continuing discoveries on the complexity and diversity of the DC network make it necessary to reassess current knowledge of DC subsets in the establishment of T cell tolerance. In particular, markers historically attributed to DCs, such as MHC-II and the integrin CD11c, do not actually discriminate among DC subpopulations and often overlap with additional members of the mononuclear phagocyte system, including monocytes and macrophages, especially under inflammatory conditions (44). Through the discovery of specific DC markers and a review of DC lineages based on ontogeny, researchers will be able to better understand the contribution of DC subtypes to Treg development in a variety of scenarios.

CD8α$^+$/CD103$^+$ cDCs

Conventional DCs expressing the CD8α$^+$ marker can be found in lymphoid organs while cDCs located in non-lymphoid tissues express the marker CD103$^+$, with the exception of a subpopulation of DCs in the intestine (5, 7, 45, 46). The CD8α$^+$/CD103$^+$ subsets share a common origin and function and depend on the transcription factors Batf3, IRF8, and Id2 for development (6, 8, 44, 46–48). Given the ability of these subsets to cross-present antigens, CD8α$^+$ and CD103$^+$ DCs are traditionally associated with the cross-priming of pathogens- and tumor-derived antigens to CD8$^+$ T cells (5). Moreover, in the absence of inflammation, CD8α$^+$/CD103$^+$ DCs can also regulate the induction of Tregs and mediate T cell tolerance (8, 21). Functionally, splenic CD8α$^+$ DCs are superior at inducing Foxp3$^+$ T cell differentiation than CD8α$^-$ DC counterparts due to their ability to produce transforming growth factor beta (TGF-β) (39, 49). Furthermore, CD8α$^+$ cells have demonstrated proficient abilities to induce iTregs in *in vivo* studies of antigen targeting via the expression of antibodies specific to endocytic receptors, such as the C-type lectins DEC205 and DNGR-1 (50, 51). These results indicate that CD8α$^+$ DCs can be manipulated to induce iTreg differentiation *in vivo* by means of antigen delivery.

In tissues, CD8α$^+$/CD103$^+$ DCs related to the gut-associated lymphoid tissue (GALT) are critically involved in oral tolerance and in inducing the *de novo* differentiation of Foxp3$^+$ T cells (52). However, the ability of CD8α$^+$/CD103$^+$ DCs to induce iTregs *in vivo* is not exclusive, as indicated by studies in *batf3*$^{-/-}$ mice, which lack the CD8α$^+$/CD103$^+$ DC subsets but display normal frequencies of intestinal Tregs, indicating that additional APCs can compensate to safeguard Treg homeostasis in the intestine (45).

CD11b$^+$ cDC

Conventional DCs expressing CD11b$^+$ are found across lymphoid organs and non-lymphoid tissues, but their function in tolerance has been challenging to address, partially since these cells express markers that are shared with macrophages and monocytes, such as CD11c, MHC-II, and CD11b (7). Nevertheless, in inflamed skin, dermal CD11b$^+$ DCs display a marked ability to induce Foxp3$^+$ T cells (53). Furthermore, the capacity of DCs to mediate Treg induction in the dermis is attributable to the CD11b$^+$CD103$^-$ subtype (54). These findings indicate that CD11b$^+$ DCs can regulate the homeostasis of Tregs in the skin.

Nevertheless, and as noted for CD103$^+$ DCs, the selective ablation of CD11b$^+$ DCs does not alter Treg frequencies, indicating that these cells may be sufficient but not necessary for Treg cell homeostasis (55). The discovery of new surface molecules allowing for the distinction of *bona fide* CD11b$^+$ DCs such as CD24, CD64, and MerTK will provide relevant information on this cell type in Treg induction.

Plasmacytoid DCs

Plasmacytoid dendritic cells found in the bone marrow and lymphoid organs, which express the markers CD11c, B220, Siglec-H, and BST2, depend on the E2-2 transcription factor for development (7) and produce large amounts of type I interferon in response to viral recognition (7). Although the involvement of pDCs in antigen presentation to T cells is a matter of debate (56), MHC-II-restricted antigen presentation by pDCs promotes Foxp3$^+$ T cell expansion that results in protection against experimental autoimmune encephalomyelitis (57). Furthermore, pDCs with dietary antigens can mediate oral tolerance via CD4$^+$ and CD8$^+$ T cells (58) and induce tolerance to vascularized cardiac allografts via the generation of Foxp3$^+$ T cells (59). In humans, pDCs isolated from blood induce Foxp3$^+$ T cells (60), which is

attributed to the expression of the enzyme indoleamine 2,3-dioxygenase, PD-L1, and the inducible costimulator-ligand (10, 61, 62). In addition, pDCs residing in the human thymus drive nTreg development *ex vivo* (63), and stimuli such as cytosine-phosphate-guanine and the thymic stromal lymphopoietin have shown to promote this function (64). These findings indicate that human pDCs have the potential to be used as tools for Treg induction. In contrast to human counterparts, murine pDCs display limited abilities to induce Tregs (10). On the other hand, antigen targeting to the human BDCA-2 molecule in B6.BDCA2 transgenic mice results in antigen-specific tolerance and an increased Tregs/T effector ratio (65).

Altogether, current evidence indicates that pDCs strongly contribute to Treg homeostasis and could be attractive candidates for immunotherapy treatments against several conditions, such as transplant rejection, autoimmune diseases, and cancer (66).

Inflammatory DCs

Inflammatory dendritic cells present during infection/inflammation, are derived from monocytes, and express the markers CD11c, MHC-II, CD11b, and CD64. iDCs release large amounts of inflammatory cytokines upon pathogen recognition, including TNF and the inducible nitric oxide synthase (7). Moreover, iDCs drive Th1- and Th17-mediated immunity, a feature also recorded in humans (67). Given that these cells are not present in steady-state conditions, their role in maintaining Treg homeostasis *in vivo* is not fully understood. Nevertheless, *in vitro* models of DC generation have been widely used. DCs stimulated by the granulocyte macrophage colony-stimulating factor (GM-CSF), plus or minus interleukin (IL)-4, induce large numbers of cells that resemble those originated under inflammatory contexts (8, 68), and these cells have been broadly used as tools to differentiate Tregs *ex vivo* (15, 69). However, recent findings indicate that GM-CSF-derived DCs are composed by a heterogeneous mixture of macrophages and DCs, which are indistinguishable through quantification of CD11c and MHC-II expression (70). It is also currently unclear which population is mediating Treg induction.

Thus, current data indicate that while DCs facilitate the induction of Tregs, there is no strict dependency on any DC lineage to sustain this function. Rather, it seems that several immune cell types, depending on the scenario, can contribute to the regulation of Treg cell homeostasis.

ROLE OF EXOGENOUS AND ENDOGENOUS TRIGGERS OF TOLEROGENIC DCs

The ability of DCs to favor Treg homeostasis and restrict T cell immunity can be fine-tuned by several extracellular and intracellular factors. These factors include microenvironmental features, the expression of surface molecules, and the selective activation of specific signaling pathways that promote Treg induction. In addition, TolDCs have been generated and studied in contexts of pharmacological modulation. The aspects that contribute to the programing of DCs in regards to triggering T cell tolerance are described below.

Microenvironment

The homeostasis of Tregs can be specifically regulated depending on location, metabolism of dietary products, or exposure to microbes or tumors (71, 72). In the microenvironment of a tumor, cancer cells can produce suppressive factors that promote DCs to induce Tregs that can, in turn, contribute to tumor progression (71). For example, DCs isolated from tumor-bearing animals are programed by cancer cells to promote Treg proliferation via TGF-β production (73). Moreover, apoptotic cells resulting from physiological processes are potent inducers of tolerance via DCs (74). Interestingly, early traslocation of annexin A1 contributes an essential tolerogenic property to apoptotic cells, conferring their ability to suppress DC activation and cellular immunity (75). Annexin A1 on apoptotic cells critically interferes with TLR signaling pathways upstream of NFκB activation and, in turn, preventing antigen-specific T cell responses (75). In addition, clearance of dead cells by DCs, which requires dedicated receptors such as DEC205 (76), results in the induction of Tregs (77). In *in vivo* models, the apoptotic cargo is preferentially captured by the $CD8\alpha^+$ lineage of DCs, which are able to mediate tolerance to cell-associated antigens (78).

Another characteristic site for Treg presence is the gut (52, 71, 72, 79, 80). Here, GALT–$CD103^+$ DCs specialize in the synthesis of retinoic acid (RA) and are the cell type predominantly responsible for Treg generation (52, 79). RA is a metabolite of vitamin A that regulates Treg induction by enhancing TGF-β-induced conversion of $CD4^+$ T cells to $Foxp3^+$ T cells (33, 81, 82). Specifically, RA sustains the differentiation of $Foxp3^+$ T cells by acting *in cis* on naïve T cells or *in trans* by relieving the inhibition exerted by $CD4^+$ memory T cells on iTreg development (33, 81, 82). $CD103^+$ DCs isolated from the small intestine lamina propria can synthesize RA and secrete bioactive TGF-β at levels sufficient to trigger Treg differentiation (52, 71). Notably, the effect of RA in promoting Tregs is not restricted to the intestine. In the skin, RA production is attributed to dermal $CD11b^+$ DCs, which trigger *de novo* iTreg differentiation with higher efficiency than $CD103^+$ counterparts (54). Even though the tissue-associated signals that lead to RA production by distinct DC subsets in the gut and skin are unclear, these findings indicate that the ability to induce iTregs is not an attribute of a dedicated DC subtype and that the outcome of the immune response is dictated by the integration of multiple environmental cues.

In a physiological context, DCs may also contribute to the maintenance of Treg homeostasis through the induction of tolerance to commensal microbiota (83, 84). Although the predominating standpoint is that DCs activated with microbes via pattern recognition receptors (PRRs) provide the signals necessary to elicit T cell immunity, it has been also reported that Tregs can arise in non-sterile conditions. This notion is supported by studies in germ-free mice, which display reduced Tregs frequency (85, 86). In fact, certain intestinal bacteria, including *Clostridium* strains and the human commensal bacteria *Bacteroides fragilis*, have been found to promote Treg development (85, 87). These findings open new avenues for studying the role of DCs in Treg homeostasis in the context of microbe

recognition, particularly against commensal and/or symbiotic microbiota. However, extensive studies must be carried out to reveal the mechanisms by which these particular microbes are able to induce Tregs and prevent T cell immunity or even Treg plasticity. Identification of these particular signatures would be greatly relevant to the advancement of DCs use in the treatment of diseases as there are additional microbial compounds, such as the fungal β-glucan curdlan, that trigger reprograming of Tregs into Th17 cells, which may result in immunity rather than tolerance depending on the conditions (88).

The *in vivo* identification of microbial-derived components that can trigger Tregs is an emerging field with many prospects related to clinical immunology. Along these lines, it remains to be determined whether the effects of commensal microbiota are indeed mediated by DC subtypes.

SURFACE MOLECULES AND ACTIVATION OF SIGNALING RESPONSES

Dendritic cell function can be modulated by the expression of surface molecules and activation of signaling cascades responsible for promoting Treg cells. In addition to the endocytic receptors such as DEC205 that can be exploited for Treg induction, certain surface molecules can confer tolerogenic properties to DCs. On one hand, expression of the TGF-β activating integrin αvβ8 by DCs is required for efficient Treg induction (89, 90). In fact, mice deficient in αvβ8 in DCs have decreased abilities to generate iTregs. This limitation causes autoimmunity and colitis in mice (89, 90), indicating that TGF-β processing by DCs is a key feature for maintaining Treg homeostasis.

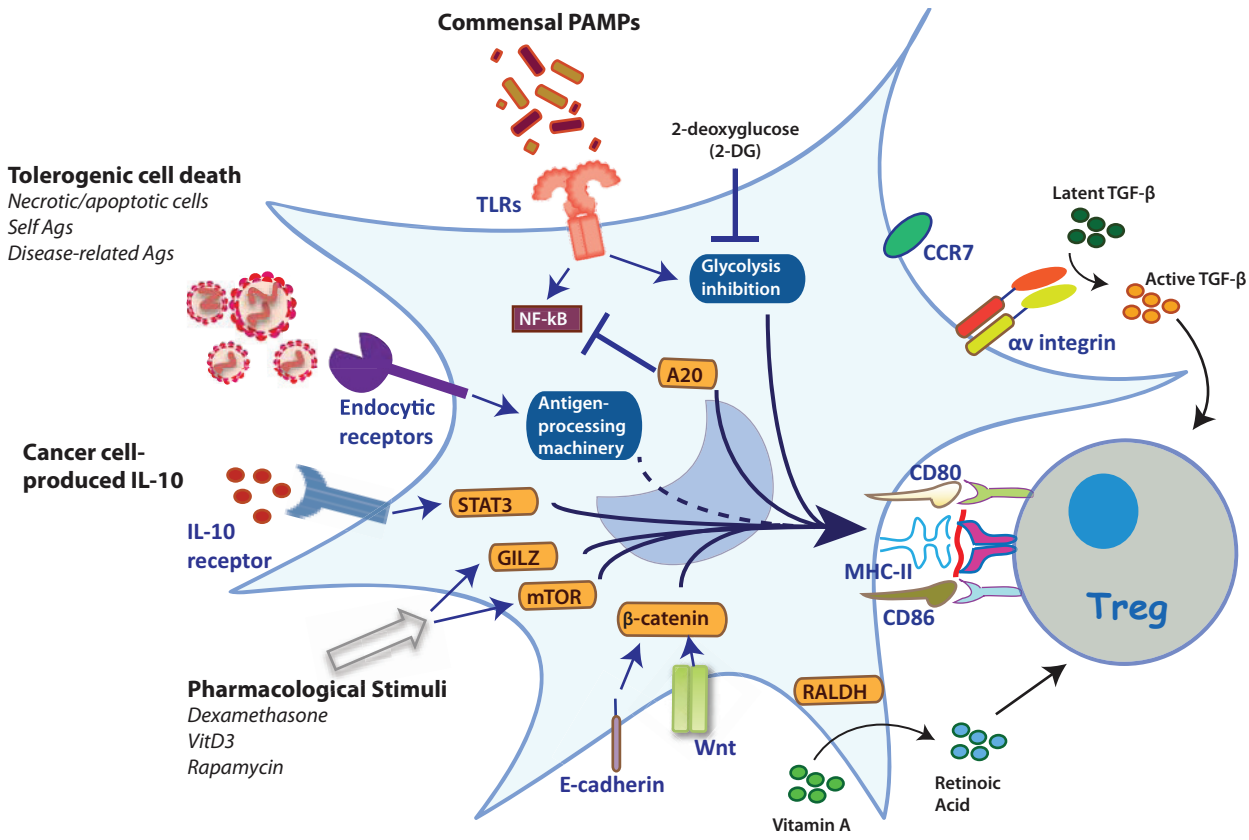

FIGURE 1 | Stimuli and mechanisms that induce the generation of TolDCs. Several stimuli can trigger different intracellular mechanisms that reprogram TolDCs to induce Tregs. TLR2 triggering results in Treg induction (91). These include activation via TLR2, expression of αv integrin for activation of TGF-β, and expression of endocytic receptors responsible to divert the antigenic cargo for presentation in tolerogenic contexts. Furthermore, the inhibition of glycolysis upon TLR stimulation by 2-deoxyglucose, promotes Treg induction (93). Moreover, the ubiquitin-editing protein A20 is a crucial regulator of TLR-driven DC activation responsible for preventing autoimmunity (94). PRR-independent mechanisms of DC activation, such as through E-cadherin and β-catenin, lead to the induction of IL-10-producing T cells with autoimmune competency (95). Likewise, expression of the retinoic-acid synthesizing enzyme 2 (RALDH2) in DCs allows the synthesis and secretion of RA, a crucial regulator of Treg homeostasis (80, 81). Furthermore, additional physiological stimuli associated with the induction of tolerance, such as apoptotic bodies, have double functions: (i) their internalization by DCs induces tolerance itself; and (ii) these are a broad source of self-antigens that can be loaded onto MHC-II molecules and presented to $CD4^+$ T cells (77). Additionally, specifically selected malignant cells produce IL-10, which is considered a mechanism of immune evasion since this cytokine inhibits MHC-I expression on cancer cells and strongly induces a tolerogenic phenotype on DCs by inducing IL-10 expression in a paracrine manner (96). Finally, pharmacological stimuli are able to trigger different transcription factors (e.g., GILZ, mTOR) that activate tolerogenic-associated protein expression on DCs (97, 98).

On the other hand, evidence indicates that signaling via PRRs in DCs also contributes to the establishment of lymphocyte tolerance via Tregs (**Figure 1**). Toll-like receptor 2 (TLR2) expression in DCs couples innate immunity to the induction of Foxp3$^+$ T cells through activation of IL-10 and the RA-metabolizing enzyme retinaldehyde dehydrogenase 2 (91). Importantly, TLR2 signaling in DCs mediates suppression against autoimmunity via the induction of Tregs (91). In addition to TLR2, additional PRRs can induce IL-10 in DCs, also leading to the induction of Treg responses. The modulation of IL-10 secretion by DCs via the DC-SIGN receptor has been associated with Foxp3$^+$ expression in naïve T cells in a murine model of nephrotoxic nephritis (92). Moreover, a monoclonal antibody to P-selectin, which also interacts with DC-SIGN, induces IL-10 secretion in human DCs that, in turn, contribute to the induction of CD4$^+$CD25$^+$Foxp3$^+$ T cells (92).

Regulators of PRR signaling in DCs can also dictate the balance between T cell tolerance and immunity. The ubiquitin-editing enzyme A20, an anti-inflammatory protein involved in the attenuation of NF-κB signaling downstream of TLRs, is a crucial regulator of immune tolerance (**Figure 1**) (94). DC-specific deletion of A20 results in autoimmunity and systemic inflammation in mice, demonstrating the role of this protein as a key regulator in DC tolerance, which may be important to consider for clinical applications.

On the other hand, DCs able to induce Tregs can also be generated in the absence of PRR engagement. For instance, the maturation of DCs in response to the disruption of cell-to-cell interactions through the blockade of E-cadherin results in the induction of Tregs (95). This form of DC activation seems partially dependent on β-catenin (99). Interestingly, β-catenin signaling in DCs may also be a key controller of tolerance (83). The expression of β-catenin in DCs regulates the expression of RA, TGF-β, and IL-10 (**Figure 1**) (83). Importantly, selective ablation of β-catenin in CD11c-expressing cells leads to reduced Foxp3$^+$ Tregs in the intestine (83), highlighting the contribution of specific signaling pathways in programing TolDCs. In this context, there are ongoing efforts aiming to identify gene expression signatures that distinguish between PRR-activated DCs and mature homeostatic DCs. These studies may shed light on the regulatory gene networks relevant to TolDC programing (5).

Besides changes in gene expression, the process of DC activation also involves a metabolic reprograming required to elicit T cell immunity. This process includes a rapid increase in glycolysis, which is essential to fulfill the biosynthetic and bioenergetic demands of activated DCs (93, 100). Inhibition of glycolysis in activated DCs through 2-deoxyglucose, an inhibitor of hexokinase, biases DCs to induce Foxp3$^+$ T cells (93). This observation suggests that gaining better insights into the metabolic programs of steady-state versus activated DCs may be useful toward ultimately programing TolDCs for therapeutic use. In humans, TolDCs are characterized by increased catabolic pathway signaling, oxidative phosphorylation, mitochondrial oxidative activity, and fatty acid oxidation, as compared to activated DCs. In fact, the inhibition of fatty acid oxidation prevents the functioning of TolDCs and partially restores T cell stimulatory capacity (101). These data suggest that interfering with metabolic checkpoints in DC maturation may be an interesting strategy for programing TolDCs for therapeutic purposes.

PHARMACOLOGICAL MODULATION OF TolDCs

The phenotypic plasticity of DCs can be modulated by a variety of stimuli with different nature and origin (**Figure 1**). Although there is currently no absolute consensus about the phenotype and characteristics of TolDCs, several studies support the notion that these cells must be able to induce a Foxp3$^+$ Treg phenotype from naïve T cells (83, 84, 102, 103). From a clinical perspective, DC activation can be pharmacologically altered to obtain DCs capable of mediating T cell tolerance. Several cytokines, anti-inflammatory, and immunosuppressive drugs trigger a tolerogenic phenotype by interfering with checkpoints of DC differentiation and activation. These include glucocorticoids, vitamin D, aspirin, rapamycin, rosiglitazone, heme oxygenase-1 inducers, and andrographolide, among others (104–112). In addition, the ability of different cytokines to generate TolDCs has been tested, with IL-10 and TGF-β found as the most effective factors. DCs cultured in the presence of IL-10 and or TGF-β trigger anergy in naïve and memory T cells and are also able to activate T cells with an IL-2^{low}, IFN-γ^{low}, and IL-10^{high} Foxp3$^+$ profile (10, 113). In fact, IL-10 is also involved in the differentiation of Foxp3$^+$ Tregs in human *in vitro* models (11).

Regarding the pharmacological modulation of DCs in human, different protocols have been developed by adapting strategies used in mouse cells. Dexamethasone, vitamin D3, and rapamycin, in conjunction with the GM-CSF and IL-4, are the most used reagents in the *ex vivo* differentiation of peripheral blood monocytes into TolDCs (114). Dexamethasone, a glucocorticoid widely used to treat autoimmune diseases and graft rejection, mantains an immature DC phenotype that is associated with low expression of human leukocyte antigen II and co-stimulatory molecules. In addition, dexamethasone-treated DCs maintain expression of the chemokine receptor CCR7, suggesting that these DCs may retain migratory skills, a characteristic necessary for use in immunotherapy (115–117). Interestingly, dexamethasone stimulation in DCs also upregulates the glucocorticoid-induced leucine zipper (GILZ) protein and promotes the differentiation of antigen-specific CD25$^{(high)}$Foxp3$^+$CTLA-4/CD152$^+$ and IL-10-producing Tregs able to inhibit CD4$^+$ and CD8$^+$ T cell responses (118). In fact, GILZ expression by DCs is an important regulator of Tregs (97). CD11c-GILZ^{hi} transgenic mice, which overexpress GILZ in the CD11c compartment, display an accumulation of Foxp3$^+$ Tregs in spleen and in central and peripheral lymphoid organs of aged animals (97).

For its part, vitamin D3 (1,25-dihydroxyvitamin D_3) promotes a tolerogenic phenotype in human monocyte-derived DCs via the activation of glucose metabolism. This effect is mediated by the PI3K/Akt/mTOR pathway, which controls the induction and maintenance of tolerogenic functions in DCs, stimulating the generation of Tregs (98, 119). In turn, rapamycin, an antibiotic of the macrolide family that possesses immunosuppressive properties, inhibits the maturation and effector functions of DCs. Indeed,

murine rapamycin-treated DCs are inferior stimulators of syngeneic T cells that do not alter antigen uptake or *in vivo* homing to lymphoid tissue (111). Additionally, rapamycin treatment inhibits the ability of DCs to produce IL-12 and TNFα and can induce the proliferation of $CD4^+CD25^+Foxp3^+$ Tregs (108, 120).

Taken together, the current evidence strongly supports that TolDCs generated by a single or combination of drugs could be an effective therapeutic approach to induce antigen specific Tregs in a variety of scenarios. In this regard, IL-10 and rapamycin appear to be the most suitable agents for generating TolDCs that could be applied in the treatment of human diseases.

DC-BASED IMMUNOTHERAPY AND THE INDUCTION OF TOLERANCE: WHERE ARE WE?

Over the last decade, different DC-based immunotherapies have been used to stimulate immune responses, and some have shown objective clinical benefits in patients with different types of cancer (121–125). Currently, several immunotherapeutic approaches are being studied, including for autoimmune conditions, allergies, and transplant rejection. All of these pathologies require permanent induction of T cell tolerance, as triggered by TolDC and orchestrated by proficient Treg functions. Indeed, the main target of TolDC immunotherapy should be the induction of tissue/organ infiltrating Tregs. Clinical data on TolDC-based immunotherapies remain limited, and the majority of studies are in preclinical stages. These studies focus on the development of safe and well-tolerated TolDCs with an anti-inflammatory phenotype and a preferential capacity to induce Tregs (126–128).

Regarding allergy treatments, atopic diseases are largely dominated by T helper type 2 immune responses that lead to an accumulation of eosinophils, immunoglobulin E production, and the sensitization of tissue mast cells (129). In *ex vivo* models, TolDCs… have shown to suppress the T helper type 2 immune response by inducing/expanding IL-10-expressing $CD25^+Foxp3^+LAG\text{-}3^+CTLA\text{-}4^+$ Tregs (130). Moreover, *in vitro* dexamethasone-induced human TolDCs are able to generate IL-10-expressing T cells specific for the Hev b5 antigen, a principal latex allergen (128).

On the other hand, autoimmune diseases are an issue of public health due to their heterogeneity and diverse clinical manifestations. Several studies in murine models of rheumatoid arthritis have demonstrated the efficacy of TNF-, IL-10-, and dexamethasone-treated TolDCs to treat collagen-induced arthritis, an experimental model for inflammatory joint diseases (131–133). In fact, the first prospective phase I clinical trial in patients with rheumatoid arthritis was recently concluded (134). In this trial, patients treated with autologous modified TolDCs and pulsed with citrullinated peptide antigens showed changes in the Treg/T effector ratio, where in 11 out of 15 treated patients, there was at least a 25% reduction in $CD4^+CD25^+CD127^+$ T effector cells. However, Tregs increased by ≥25% in only 5 out of 15 treated patients (134). Importantly, this clinical trial has demonstrated the safety of this therapeutic approach as well as its effects on patients' immunological parameters.

CONCLUSION

In conclusion, and despite the substantial advances made in recent years regarding the development and function of TolDCs, it remains to be determined whether this knowledge can be applicable to the clinic. The ultimate success of TolDC-based therapies will be dictated by gaining in-depth understanding of the mechanisms by which TolDCs suppress autoreactive lymphocytes and induce Tregs; and by obtaining better insights regarding the mechanisms by TolDC suppress autoimmunity, allergies, and transplant rejection. These aspects include the stability/commitment of generated TolDC, especially in an *in vivo* inflammatory milieu, among others.

In addition, as there is no current consensus about which stimuli would be more reliable in inducing a committed TolDC phenotype, it is highly plausible that specific combination of these triggers, or even an additional stimuli yet to be identified, are needed to reach this phenotype. A third aspect to be addressed is the clear identification of specific receptors and the downstream signaling pathways that can be triggered in DCs to induce Treg responses. In this context, studies using high-throughput approaches (e.g., proteomics and genomics) will be of great help to identify the crucial genes/proteins networks involved in programing TolDCs suitable for Treg induction. This knowledge will assuredly facilitate the near-future design of reliable and clinically effective TolDCs for the treatment of several human diseases and conditions.

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REFERENCES

1. Steinman RM. Decisions about dendritic cells: past, present, and future. *Annu Rev Immunol* (2012) **30**:1–22. doi:10.1146/annurev-immunol-100311-102839
2. Steinman RM, Idoyaga J. Features of the dendritic cell lineage. *Immunol Rev* (2010) **234**(1):5–17. doi:10.1111/j.0105-2896.2009.00888.x
3. Cheong C, Matos I, Choi JH, Dandamudi DB, Shrestha E, Longhi MP, et al. Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. *Cell* (2010) **143**(3):416–29. doi:10.1016/j.cell.2010.09.039
4. Lewis KL, Reizis B. Dendritic cells: arbiters of immunity and immunological tolerance. *Cold Spring Harb Perspect Biol* (2012) **4**(8):a007401. doi:10.1101/cshperspect.a007401
5. Dalod M, Chelbi R, Malissen B, Lawrence T. Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming. *EMBO J* (2014) **33**(10):1104–16. doi:10.1002/embj.201488027
6. Belz GT, Nutt SL. Transcriptional programming of the dendritic cell network. *Nat Rev Immunol* (2012) **12**(2):101–13. doi:10.1038/nri3149
7. Schlitzer A, Ginhoux F. Organization of the mouse and human DC network. *Curr Opin Immunol* (2014) **26**:90–9. doi:10.1016/j.coi.2013.11.002

8. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* (2013) **31**:563–604. doi:10.1146/annurev-immunol-020711-074950
9. Fuchsberger M, Hochrein H, O'Keeffe M. Activation of plasmacytoid dendritic cells. *Immunol Cell Biol* (2005) **83**(5):571–7. doi:10.1111/j.1440-1711.2005.01392.x
10. Guery L, Hugues S. Tolerogenic and activatory plasmacytoid dendritic cells in autoimmunity. *Front Immunol* (2013) **4**:59. doi:10.3389/fimmu.2013.00059
11. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol* (2011) **11**(11):762–74. doi:10.1038/nri3070
12. Caminschi I, Maraskovsky E, Heath WR. Targeting dendritic cells in vivo for cancer therapy. *Front Immunol* (2012) **3**:13. doi:10.3389/fimmu.2012.00013
13. Steinman RM, Nussenzweig MC. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci U S A* (2002) **99**(1):351–8. doi:10.1073/pnas.231606698
14. Ueno H, Schmitt N, Klechevsky E, Pedroza-Gonzalez A, Matsui T, Zurawski G, et al. Harnessing human dendritic cell subsets for medicine. *Immunol Rev* (2010) **234**(1):199–212. doi:10.1111/j.0105-2896.2009.00884.x
15. Yamazaki S, Iyoda T, Tarbell K, Olson K, Velinzon K, Inaba K, et al. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* (2003) **198**(2):235–47. doi:10.1084/jem.20030422
16. Yamazaki S, Inaba K, Tarbell KV, Steinman RM. Dendritic cells expand antigen-specific Foxp3+ CD25+ CD4+ regulatory T cells including suppressors of alloreactivity. *Immunol Rev* (2006) **212**:314–29. doi:10.1111/j.0105-2896.2006.00422.x
17. Tarbell KV, Petit L, Zuo X, Toy P, Luo X, Mqadmi A, et al. Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. *J Exp Med* (2007) **204**(1):191–201. doi:10.1084/jem.20061631
18. Sela U, Olds P, Park A, Schlesinger SJ, Steinman RM. Dendritic cells induce antigen-specific regulatory T cells that prevent graft versus host disease and persist in mice. *J Exp Med* (2011) **208**(12):2489–96. doi:10.1084/jem.20110466
19. Scheinecker C, McHugh R, Shevach EM, Germain RN. Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. *J Exp Med* (2002) **196**(8):1079–90. doi:10.1084/jem.20020991
20. Matheu MP, Othy S, Greenberg ML, Dong TX, Schuijs M, Deswarte K, et al. Imaging regulatory T cell dynamics and CTLA4-mediated suppression of T cell priming. *Nat Commun* (2015) **6**:6219. doi:10.1038/ncomms7219
21. Hsieh C-S, Lee H-M, Lio C-WJ. Selection of regulatory T cells in the thymus. *Nat Rev Immunol* (2012) **12**(3):157–67. doi:10.1038/nri3155
22. Ganguly D, Haak S, Sisirak V, Reizis B. The role of dendritic cells in autoimmunity. *Nat Rev Immunol* (2013) **13**(8):566–77. doi:10.1038/nri3477
23. Ohnmacht C, Pullner A, King SBS, Drexler I, Meier S, Brocker T, et al. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *J Exp Med* (2009) **206**(3):549–59. doi:10.1084/jem.20082394
24. Birnberg T, Bar-On L, Sapoznikov A, Caton ML, Cervantes-Barragán L, Makia D, et al. Lack of conventional dendritic cells is compatible with normal development and T cell homeostasis, but causes myeloid proliferative syndrome. *Immunity* (2008) **29**(6):986–97. doi:10.1016/j.immuni.2008.10.012
25. Proietto AI, van Dommelen S, Zhou P, Rizzitelli A, D'Amico A, Steptoe RJ, et al. Dendritic cells in the thymus contribute to T-regulatory cell induction. *Proc Natl Acad Sci U S A* (2008) **105**(50):19869–74. doi:10.1073/pnas.0810268105
26. Perry JSA, Lio C-WJ, Kau AL, Nutsch K, Yang Z, Gordon JI, et al. Distinct contributions of aire and antigen-presenting-cell subsets to the generation of self-tolerance in the thymus. *Immunity* (2014) **41**(3):414–26. doi:10.1016/j.immuni.2014.08.007
27. Coquet JM, Ribot JC, Babała N, Middendorp S, van der Horst G, Xiao Y, et al. Epithelial and dendritic cells in the thymic medulla promote CD4+Foxp3+ regulatory T cell development via the CD27-CD70 pathway. *J Exp Med* (2013) **210**(4):715–28. doi:10.1084/jem.20112061
28. Darrasse-Jèze G, Deroubaix S, Mouquet H, Victora GD, Eisenreich T, Yao K-H, et al. Feedback control of regulatory T cell homeostasis by dendritic cells in vivo. *J Exp Med* (2009) **206**(9):1853–62. doi:10.1084/jem.20090746
29. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* (2007) **8**(2):191–7. doi:10.1038/ni1428
30. Liu K, Victora GD, Schwickert TA, Guermonprez P, Meredith MM, Yao K, et al. In vivo analysis of dendritic cell development and homeostasis. *Science* (2009) **324**(5925):392–7. doi:10.1126/science.1170540
31. Swee LK, Bosco N, Malissen B, Ceredig R, Rolink A. Expansion of peripheral naturally occurring T regulatory cells by Fms-like tyrosine kinase 3 ligand treatment. *Blood* (2009) **113**(25):6277–87. doi:10.1182/blood-2008-06-161026
32. Bar-On L, Birnberg T, Kim KW, Jung S. Dendritic cell-restricted CD80/86 deficiency results in peripheral regulatory T-cell reduction but is not associated with lymphocyte hyperactivation. *Eur J Immunol* (2011) **41**(2):291–8. doi:10.1002/eji.201041169
33. Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med* (2007) **204**(8):1765–74. doi:10.1084/jem.20070719
34. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* (2000) **12**(4):431–40. doi:10.1016/S1074-7613(00)80195-8
35. Tang Q, Henriksen KJ, Boden EK, Tooley AJ, Ye J, Subudhi SK, et al. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* (2003) **171**(7):3348–52. doi:10.4049/jimmunol.171.7.3348
36. Liang S, Alard P, Zhao Y, Parnell S, Clark SL, Kosiewicz MM. Conversion of CD4+ CD25- cells into CD4+ CD25+ regulatory T cells in vivo requires B7 costimulation, but not the thymus. *J Exp Med* (2005) **201**(1):127–37. doi:10.1084/jem.20041201
37. Lopez MN, Pesce B, Kurte M, Perez C, Segal G, Roa J, et al. A synthetic peptide homologous to IL-10 functional domain induces monocyte differentiation to TGF-beta+ tolerogenic dendritic cells. *Immunobiology* (2011) **216**(10):1117–26. doi:10.1016/j.imbio.2011.04.006
38. Caton ML, Smith-Raska MR, Reizis B. Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J Exp Med* (2007) **204**(7):1653–64. doi:10.1084/jem.20062648
39. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. *Proc Natl Acad Sci U S A* (2008) **105**(27):9331–6. doi:10.1073/pnas.0710441105
40. Yogev N, Frommer F, Lukas D, Kautz-Neu K, Karram K, Ielo D, et al. Dendritic cells ameliorate autoimmunity in the CNS by controlling the homeostasis of PD-1 receptor(+) regulatory T cells. *Immunity* (2012) **37**(2):264–75. doi:10.1016/j.immuni.2012.05.025
41. Vitali C, Mingozzi F, Broggi A, Barresi S, Zolezzi F, Bayry J, et al. Migratory, and not lymphoid-resident, dendritic cells maintain peripheral self-tolerance and prevent autoimmunity via induction of iTreg cells. *Blood* (2012) **120**(6):1237–45. doi:10.1182/blood-2011-09-379776
42. Meredith MM, Liu K, Darrasse-Jèze G, Kamphorst AO, Schreiber HA, Guermonprez P, et al. Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *J Exp Med* (2012) **209**(6):1153–65. doi:10.1084/jem.20112675
43. Schraml BU, van Blijswijk J, Zelenay S, Whitney PG, Filby A, Acton SE, et al. Genetic tracing via DNGR-1 expression history defines dendritic cells as a hematopoietic lineage. *Cell* (2013) **154**(4):843–58. doi:10.1016/j.cell.2013.07.014
44. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, et al. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat Rev Immunol* (2014) **14**(8):571–8. doi:10.1038/nri3712
45. Edelson BT, Kc W, Juang R, Kohyama M, Benoit LA, Klekotka PA, et al. Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. *J Exp Med* (2010) **207**(4):823–36. doi:10.1084/jem.20091627
46. Schraml BU, Reis ESC. Defining dendritic cells. *Curr Opin Immunol* (2015) **32C**:13–20. doi:10.1016/j.coi.2014.11.001
47. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, et al. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* (2008) **322**(5904):1097–100. doi:10.1126/science.1164206

48. Ginhoux F, Liu K, Helft J, Bogunovic M, Greter M, Hashimoto D, et al. The origin and development of nonlymphoid tissue CD103+ DCs. *J Exp Med* (2009) **206**(13):3115–30. doi:10.1084/jem.20091756
49. Yamazaki S, Dudziak D, Heidkamp GF, Fiorese C, Bonito AJ, Inaba K, et al. CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *J Immunol* (2008) **181**(10):6923–33. doi:10.4049/jimmunol.181.10.6923
50. Mahnke K, Qian Y, Knop J, Enk AH. Induction of CD4+/CD25+ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood* (2003) **101**(12):4862–9. doi:10.1182/blood-2002-10-3229
51. Joffre OP, Sancho D, Zelenay S, Keller AM, Reis e Sousa C. Efficient and versatile manipulation of the peripheral CD4+ T-cell compartment by antigen targeting to DNGR-1/CLEC9A. *Eur J Immunol* (2010) **40**(5):1255–65. doi:10.1002/eji.201040419
52. Coombes JL, Siddiqui KRR, Arancibia-Cárcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* (2007) **204**(8):1757–64. doi:10.1084/jem.20070590
53. McLachlan JB, Catron DM, Moon JJ, Jenkins MK. Dendritic cell antigen presentation drives simultaneous cytokine production by effector and regulatory T cells in inflamed skin. *Immunity* (2009) **30**(2):277–88. doi:10.1016/j.immuni.2008.11.013
54. Guilliams M, Crozat K, Henri S, Tamoutounour S, Grenot P, Devilard E, et al. Skin-draining lymph nodes contain dermis-derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells. *Blood* (2010) **115**(10):1958–68. doi:10.1182/blood-2009-09-245274
55. Lewis KL, Caton ML, Bogunovic M, Greter M, Grajkowska LT, Ng D, et al. Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity* (2011) **35**(5):780–91. doi:10.1016/j.immuni.2011.08.013
56. Villadangos JA, Young L. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity* (2008) **29**(3):352–61. doi:10.1016/j.immuni.2008.09.002
57. Irla M, Küpfer N, Suter T, Lissilaa R, Benkhoucha M, Skupsky J, et al. MHC class II-restricted antigen presentation by plasmacytoid dendritic cells inhibits T cell-mediated autoimmunity. *J Exp Med* (2010) **207**(9):1891–905. doi:10.1084/jem.20092627
58. Goubier A, Dubois B, Gheit H, Joubert G, Villard-Truc F, Asselin-Paturel C, et al. Plasmacytoid dendritic cells mediate oral tolerance. *Immunity* (2008) **29**(3):464–75. doi:10.1016/j.immuni.2008.06.017
59. Ochando JC, Homma C, Yang Y, Hidalgo A, Garin A, Tacke F, et al. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* (2006) **7**(6):652–62. doi:10.1038/ni1333
60. Moseman EA, Liang X, Dawson AJ, Panoskaltsis-Mortari A, Krieg AM, Liu Y-J, et al. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J Immunol* (2004) **173**(7):4433–42. doi:10.4049/jimmunol.173.7.4433
61. Puccetti P, Fallarino F. Generation of T cell regulatory activity by plasmacytoid dendritic cells and tryptophan catabolism. *Blood Cells Mol Dis* (2008) **40**(1):101–5. doi:10.1016/j.bcmd.2007.06.026
62. Conrad C, Gregorio J, Wang YH, Ito T, Meller S, Hanabuchi S, et al. Plasmacytoid dendritic cells promote immunosuppression in ovarian cancer via ICOS costimulation of Foxp3(+) T-regulatory cells. *Cancer Res* (2012) **72**(20):5240–9. doi:10.1158/0008-5472.CAN-12-2271
63. Martin-Gayo E, Sierra-Filardi E, Corbi AL, Toribio ML. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood* (2010) **115**(26):5366–75. doi:10.1182/blood-2009-10-248260
64. Hanabuchi S, Ito T, Park WR, Watanabe N, Shaw JL, Roman E, et al. Thymic stromal lymphopoietin-activated plasmacytoid dendritic cells induce the generation of FOXP3+ regulatory T cells in human thymus. *J Immunol* (2010) **184**(6):2999–3007. doi:10.4049/jimmunol.0804106
65. Chappell CP, Giltiay NV, Draves KE, Chen C, Hayden-Ledbetter MS, Shlomchik MJ, et al. Targeting antigens through blood dendritic cell antigen 2 on plasmacytoid dendritic cells promotes immunologic tolerance. *J Immunol* (2014) **192**(12):5789–801. doi:10.4049/jimmunol.1303259
66. Matta BM, Castellaneta A, Thomson AW. Tolerogenic plasmacytoid DC. *Eur J Immunol* (2010) **40**(10):2667–76. doi:10.1002/eji.201040839
67. Segura E, Touzot M, Bohineust A, Cappuccio A, Chiocchia G, Hosmalin A, et al. Human inflammatory dendritic cells induce Th17 cell differentiation. *Immunity* (2013) **38**(2):336–48. doi:10.1016/j.immuni.2012.10.018
68. Xu Y, Zhan Y, Lew AM, Naik SH, Kershaw MH. Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J Immunol* (2007) **179**(11):7577–84. doi:10.4049/jimmunol.179.11.7577
69. Pen JJ, De Keersmaecker B, Maenhout SK, Van Nuffel AMT, Heirman C, Corthals J, et al. Modulation of regulatory T cell function by monocyte-derived dendritic cells matured through electroporation with mRNA encoding CD40 ligand, constitutively active TLR4, and CD70. *J Immunol* (2013) **191**(4):1976–83. doi:10.4049/jimmunol.1201008
70. Helft J, Bottcher J, Chakravarty P, Zelenay S, Huotari J, Schraml BU, et al. GM-CSF mouse bone marrow cultures comprise a heterogeneous population of CD11c(+)MHCII(+) macrophages and dendritic cells. *Immunity* (2015) **42**(6):1197–211. doi:10.1016/j.immuni.2015.05.018
71. Belkaid Y, Oldenhove G. Tuning microenvironments: induction of regulatory T cells by dendritic cells. *Immunity* (2008) **29**(3):362–71. doi:10.1016/j.immuni.2008.08.005
72. Grainger JR, Hall JA, Bouladoux N, Oldenhove G, Belkaid Y. Microbe-dendritic cell dialog controls regulatory T-cell fate. *Immunol Rev* (2010) **234**(1):305–16. doi:10.1111/j.0105-2896.2009.00880.x
73. Ghiringhelli F, Puig PE, Roux S, Parcellier A, Schmitt E, Solary E, et al. Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. *J Exp Med* (2005) **202**(7):919–29. doi:10.1084/jem.20050463
74. Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. *Nat Rev Immunol* (2014) **14**(3):166–80. doi:10.1038/nri3607
75. Weyd H, Abeler-Dorner L, Linke B, Mahr A, Jahndel V, Pfrang S, et al. Annexin A1 on the surface of early apoptotic cells suppresses CD8+ T cell immunity. *PLoS One* (2013) **8**(4):e62449. doi:10.1371/journal.pone.0062449
76. Shrimpton RE, Butler M, Morel AS, Eren E, Hue SS, Ritter MA. CD205 (DEC-205): a recognition receptor for apoptotic and necrotic self. *Mol Immunol* (2009) **46**(6):1229–39. doi:10.1016/j.molimm.2008.11.016
77. Kushwah R, Oliver JR, Zhang J, Siminovitch KA, Hu J. Apoptotic dendritic cells induce tolerance in mice through suppression of dendritic cell maturation and induction of antigen-specific regulatory T cells. *J Immunol* (2009) **183**(11):7104–18. doi:10.4049/jimmunol.0900824
78. Qiu CH, Miyake Y, Kaise H, Kitamura H, Ohara O, Tanaka M. Novel subset of CD8{alpha}+ dendritic cells localized in the marginal zone is responsible for tolerance to cell-associated antigens. *J Immunol* (2009) **182**(7):4127–36. doi:10.4049/jimmunol.0803364
79. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* (2007) **204**(8):1775–85. doi:10.1084/jem.20070602
80. Mucida D, Pino-Lagos K, Kim G, Nowak E, Benson MJ, Kronenberg M, et al. Retinoic acid can directly promote TGF-β-mediated Foxp3+ Treg cell conversion of naive T cells. *Immunity* (2009) **30**(4):471–2. doi:10.1016/j.immuni.2009.03.008
81. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* (2007) **317**(5835):256–60. doi:10.1126/science.1145697
82. Hill JA, Hall JA, Sun CM, Cai Q, Ghyselinck N, Chambon P, et al. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi cells. *Immunity* (2008) **29**(5):758–70. doi:10.1016/j.immuni.2008.09.018
83. Manicassamy S, Reizis B, Ravindran R, Nakaya H, Salazar-Gonzalez RM, Wang YC, et al. Activation of beta-catenin in dendritic cells regulates immunity versus tolerance in the intestine. *Science* (2010) **329**(5993):849–53. doi:10.1126/science.1188510
84. Swafford D, Manicassamy S. Wnt signaling in dendritic cells: its role in regulation of immunity and tolerance. *Discov Med* (2015) **19**(105):303–10.
85. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science* (2011) **331**(6015):337–41. doi:10.1126/science.1198469

86. Ivanov II, Honda K. Intestinal commensal microbes as immune modulators. *Cell Host Microbe* (2012) **12**(4):496–508. doi:10.1016/j.chom.2012.09.009
87. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A* (2010) **107**(27):12204–9. doi:10.1073/pnas.0909122107
88. Osorio F, LeibundGut-Landmann S, Lochner M, Lahl K, Sparwasser T, Eberl G, et al. DC activated via dectin-1 convert Treg into IL-17 producers. *Eur J Immunol* (2008) **38**(12):3274–81. doi:10.1002/eji.200838950
89. Lacy-Hulbert A, Smith AM, Tissire H, Barry M, Crowley D, Bronson RT, et al. Ulcerative colitis and autoimmunity induced by loss of myeloid alphav integrins. *Proc Natl Acad Sci U S A* (2007) **104**(40):15823–8. doi:10.1073/pnas.0707421104
90. Travis MA, Reizis B, Melton AC, Masteller E, Tang Q, Proctor JM, et al. Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. *Nature* (2007) **449**(7160):361–5. doi:10.1038/nature06110
91. Manicassamy S, Ravindran R, Deng J, Oluoch H, Denning TL, Kasturi SP, et al. Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. *Nat Med* (2009) **15**(4):401–9. doi:10.1038/nm.1925
92. Cai M, Wu J, Mao C, Ren J, Li P, Li X, et al. A Lectin-EGF antibody promotes regulatory T cells and attenuates nephrotoxic nephritis via DC-SIGN on dendritic cells. *J Transl Med* (2013) **11**:103. doi:10.1186/1479-5876-11-103
93. Everts B, Amiel E, Huang SC, Smith AM, Chang CH, Lam WY, et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. *Nat Immunol* (2014) **15**(4):323–32. doi:10.1038/ni.2833
94. Kool M, van Loo G, Waelput W, De Prijck S, Muskens F, Sze M, et al. The ubiquitin-editing protein A20 prevents dendritic cell activation, recognition of apoptotic cells, and systemic autoimmunity. *Immunity* (2011) **35**(1):82–96. doi:10.1016/j.immuni.2011.05.013
95. Jiang A, Bloom O, Ono S, Cui W, Unternaehrer J, Jiang S, et al. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. *Immunity* (2007) **27**(4):610–24. doi:10.1016/j.immuni.2007.08.015
96. Salazar-Onfray F. Interleukin-10: a cytokine used by tumors to escape immunosurveillance. *Med Oncol* (1999) **16**(2):86–94. doi:10.1007/BF02785841
97. Calmette J, Ellouze M, Tran T, Karaki S, Ronin E, Capel F, et al. Glucocorticoid-induced leucine zipper enhanced expression in dendritic cells is sufficient to drive regulatory T cells expansion in vivo. *J Immunol* (2014) **193**(12):5863–72. doi:10.4049/jimmunol.1400758
98. Ferreira GB, Vanherwegen AS, Eelen G, Gutierrez AC, Van Lommel L, Marchal K, et al. Vitamin D3 induces tolerance in human dendritic cells by activation of intracellular metabolic pathways. *Cell Rep* (2015) **10**:711–25. doi:10.1016/j.celrep.2015.01.013
99. Pulendran B. The varieties of immunological experience: of pathogens, stress, and dendritic cells. *Annu Rev Immunol* (2015) **33**:563–606. doi:10.1146/annurev-immunol-020711-075049
100. Pearce EJ, Everts B. Dendritic cell metabolism. *Nat Rev Immunol* (2015) **15**(1):18–29. doi:10.1038/nri3771
101. Malinarich F, Duan K, Hamid RA, Bijin A, Lin WX, Poidinger M, et al. High mitochondrial respiration and glycolytic capacity represent a metabolic phenotype of human tolerogenic dendritic cells. *J Immunol* (2015) **194**(11):5174–86. doi:10.4049/jimmunol.1303316
102. Maldonado RA, von Andrian UH. How tolerogenic dendritic cells induce regulatory T cells. *Adv Immunol* (2010) **108**:111–65. doi:10.1016/B978-0-12-380995-7.00004-5
103. Unger WW, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur J Immunol* (2009) **39**(11):3147–59. doi:10.1002/eji.200839103
104. Moser M. Dendritic cells in immunity and tolerance-do they display opposite functions? *Immunity* (2003) **19**(1):5–8. doi:10.1016/S1074-7613(03)00182-1
105. Rea D, van Kooten C, van Meijgaarden KE, Ottenhoff TH, Melief CJ, Offringa R. Glucocorticoids transform CD40-triggering of dendritic cells into an alternative activation pathway resulting in antigen-presenting cells that secrete IL-10. *Blood* (2000) **95**(10):3162–7.
106. Hackstein H, Morelli AE, Larregina AT, Ganster RW, Papworth GD, Logar AJ, et al. Aspirin inhibits in vitro maturation and in vivo immunostimulatory function of murine myeloid dendritic cells. *J Immunol* (2001) **166**(12):7053–62. doi:10.4049/jimmunol.166.12.7053
107. Xing N, ML LM, Bachman LA, McKean DJ, Kumar R, Griffin MD. Distinctive dendritic cell modulation by vitamin D(3) and glucocorticoid pathways. *Biochem Biophys Res Commun* (2002) **297**(3):645–52. doi:10.1016/S0006-291X(02)02262-3
108. Hackstein H, Taner T, Zahorchak AF, Morelli AE, Logar AJ, Gessner A, et al. Rapamycin inhibits IL-4 – induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. *Blood* (2003) **101**(11):4457–63. doi:10.1182/blood-2002-11-3370
109. Iruretagoyena MI, Tobar JA, Gonzalez PA, Sepulveda SE, Figueroa CA, Burgos RA, et al. Andrographolide interferes with T cell activation and reduces experimental autoimmune encephalomyelitis in the mouse. *J Pharmacol Exp Ther* (2005) **312**(1):366–72. doi:10.1124/jpet.104.072512
110. Iruretagoyena MI, Sepulveda SE, Lezana JP, Hermoso M, Bronfman M, Gutierrez MA, et al. Inhibition of nuclear factor-kappa B enhances the capacity of immature dendritic cells to induce antigen-specific tolerance in experimental autoimmune encephalomyelitis. *J Pharmacol Exp Ther* (2006) **318**(1):59–67. doi:10.1124/jpet.106.103259
111. Taner T, Hackstein H, Wang Z, Morelli AE, Thomson AW. Rapamycin-treated, alloantigen-pulsed host dendritic cells induce ag-specific T cell regulation and prolong graft survival. *Am J Transplant* (2005) **5**(2):228–36. doi:10.1046/j.1600-6143.2004.00673.x
112. Moreau A, Hill M, Thebault P, Deschamps JY, Chiffoleau E, Chauveau C, et al. Tolerogenic dendritic cells actively inhibit T cells through heme oxygenase-1 in rodents and in nonhuman primates. *FASEB J* (2009) **23**(9):3070–7. doi:10.1096/fj.08-128173
113. Torres-Aguilar H, Aguilar-Ruiz SR, Gonzalez-Perez G, Munguia R, Bajana S, Meraz-Rios MA, et al. Tolerogenic dendritic cells generated with different immunosuppressive cytokines induce antigen-specific anergy and regulatory properties in memory CD4+ T cells. *J Immunol* (2010) **184**(4):1765–75. doi:10.4049/jimmunol.0902133
114. Naranjo-Gomez M, Raich-Regue D, Onate C, Grau-Lopez L, Ramo-Tello C, Pujol-Borrell R, et al. Comparative study of clinical grade human tolerogenic dendritic cells. *J Transl Med* (2011) **9**:89. doi:10.1186/1479-5876-9-89
115. Moser M, De Smedt T, Sornasse T, Tielemans F, Chentoufi AA, Muraille E, et al. Glucocorticoids down-regulate dendritic cell function in vitro and in vivo. *Eur J Immunol* (1995) **25**(10):2818–24. doi:10.1002/eji.1830251016
116. Piemonti L, Monti P, Allavena P, Sironi M, Soldini L, Leone BE, et al. Glucocorticoids affect human dendritic cell differentiation and maturation. *J Immunol* (1999) **162**(11):6473–81.
117. Abe M, Thomson AW. Influence of immunosuppressive drugs on dendritic cells. *Transpl Immunol* (2003) **11**(3–4):357–65. doi:10.1016/S0966-3274(03)00050-9
118. Hamdi H, Godot V, Maillot MC, Prejean MV, Cohen N, Krzysiek R, et al. Induction of antigen-specific regulatory T lymphocytes by human dendritic cells expressing the glucocorticoid-induced leucine zipper. *Blood* (2007) **110**(1):211–9. doi:10.1182/blood-2006-10-052506
119. Ferreira GB, Gysemans CA, Demengeot J, da Cunha JP, Vanherwegen AS, Overbergh L, et al. 1,25-Dihydroxyvitamin D3 promotes tolerogenic dendritic cells with functional migratory properties in NOD mice. *J Immunol* (2014) **192**(9):4210–20. doi:10.4049/jimmunol.1302350
120. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. *J Immunol* (2007) **178**(11):7018–31. doi:10.4049/jimmunol.178.11.7018
121. Lopez MN, Pereda C, Segal G, Munoz L, Aguilera R, Gonzalez FE, et al. Prolonged survival of dendritic cell-vaccinated melanoma patients correlates with tumor-specific delayed type IV hypersensitivity response and reduction of tumor growth factor beta-expressing T cells. *J Clin Oncol* (2009) **27**(6):945–52. doi:10.1200/JCO.2008.18.0794
122. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. *Nat Rev Cancer* (2012) **12**(4):265–77. doi:10.1038/nrc3258
123. Gonzalez FE, Gleisner A, Falcon-Beas F, Osorio F, Lopez MN, Salazar-Onfray F. Tumor cell lysates as immunogenic sources for cancer vaccine design. *Hum Vaccin Immunother* (2014) **10**(11):3261–9. doi:10.4161/21645515.2014.982996

124. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* (2010) **363**(5):411–22. doi:10.1056/NEJMoa1001294
125. Sheikh NA, Petrylak D, Kantoff PW, Dela Rosa C, Stewart FP, Kuan LY, et al. Sipuleucel-T immune parameters correlate with survival: an analysis of the randomized phase 3 clinical trials in men with castration-resistant prostate cancer. *Cancer Immunol Immunother* (2013) **62**(1):137–47. doi:10.1007/s00262-012-1317-2
126. Anderson AE, Swan DJ, Sayers BL, Harry RA, Patterson AM, von Delwig A, et al. LPS activation is required for migratory activity and antigen presentation by tolerogenic dendritic cells. *J Leukoc Biol* (2009) **85**(2):243–50. doi:10.1189/jlb.0608374
127. Volchenkov R, Brun JG, Jonsson R, Appel S. In vitro suppression of immune responses using monocyte-derived tolerogenic dendritic cells from patients with primary Sjogren's syndrome. *Arthritis Res Ther* (2013) **15**(5):R114. doi:10.1186/ar4294
128. Escobar A, Aguirre A, Guzman MA, Gonzalez R, Catalan D, Acuna-Castillo C, et al. Tolerogenic dendritic cells derived from donors with natural rubber latex allergy modulate allergen-specific T-cell responses and IgE production. *PLoS One* (2014) **9**(1):e85930. doi:10.1371/journal.pone.0085930
129. Boguniewicz M, Leung DY. Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol Rev* (2011) **242**(1):233–46. doi:10.1111/j.1600-065X.2011.01027.x
130. Li X, Yang A, Huang H, Zhang X, Town J, Davis B, et al. Induction of type 2 T helper cell allergen tolerance by IL-10-differentiated regulatory dendritic cells. *Am J Respir Cell Mol Biol* (2010) **42**(2):190–9. doi:10.1165/rcmb.2009-0023OC
131. van Duivenvoorde LM, Han WG, Bakker AM, Louis-Plence P, Charbonnier LM, Apparailly F, et al. Immunomodulatory dendritic cells inhibit Th1 responses and arthritis via different mechanisms. *J Immunol* (2007) **179**(3):1506–15. doi:10.4049/jimmunol.179.3.1506
132. van Duivenvoorde LM, Louis-Plence P, Apparailly F, van der Voort EI, Huizinga TW, Jorgensen C, et al. Antigen-specific immunomodulation of collagen-induced arthritis with tumor necrosis factor-stimulated dendritic cells. *Arthritis Rheum* (2004) **50**(10):3354–64. doi:10.1002/art.20513
133. Healy LJ, Collins HL, Thompson SJ. Systemic administration of tolerogenic dendritic cells ameliorates murine inflammatory arthritis. *Open Rheumatol J* (2008) **2**:71–80. doi:10.2174/1874312900802010071
134. Benham H, Nel HJ, Law SC, Mehdi AM, Street S, Ramnoruth N, et al. Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype-positive rheumatoid arthritis patients. *Sci Transl Med* (2015) **7**(290):290ra87. doi:10.1126/scitranslmed.aaa9301

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Therapeutic potential of hyporesponsive CD4$^+$ T cells in autoimmunity

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The interaction between dendritic cells (DCs) and T cells is crucial on immunity or tolerance induction. In an immature or semi-mature state, DCs induce tolerance through T-cell deletion, generation of regulatory T cells, and/or induction of T-cell anergy. Anergy is defined as an unresponsive state that retains T cells in an "off" mode under conditions in which immune activation is undesirable. This mechanism is crucial for the control of T-cell responses against self-antigens, thereby preventing autoimmunity. Tolerogenic DCs (tDCs), generated *in vitro* from peripheral blood monocytes of healthy donors or patients with autoimmune pathologies, were shown to modulate immune responses by inducing T-cell hyporesponsiveness. Animal models of autoimmune diseases confirmed the impact of T-cell anergy on disease development and progression *in vivo*. Thus, the induction of T-cell hyporesponsiveness by tDCs has become a promising immunotherapeutic strategy for the treatment of T-cell-mediated autoimmune disorders. Here, we review recent findings in the area and discuss the potential of anergy induction for clinical purposes.

Keywords: tolerogenic dendritic cells, T-cell anergy, regulatory T cells, hyporesponsiveness, immunotherapy, autoimmune diseases

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Introduction

Effective peripheral tolerance mechanisms are required to eliminate circulating autoreactive T cells and thereby prevent undesired immune responses against self-antigens. The key players in this process are dendritic cells (DCs) that induce tolerance by different control mechanisms such as T-cell deletion, generation of regulatory T cells (Tregs), and/or induction of anergy (1–3). This tolerogenic role of DCs has aroused the interest for their *ex vivo* generation and their application as therapeutic tool to restore tolerance in autoimmune conditions or allergy.

Interaction between DCs and T cells occurs through three independent signals: (i) recognition of peptide-MHC complexes presented on DCs via specific TCR on T lymphocytes, (ii) binding of costimulatory molecules expressed on DCs to their respective receptors on T cells, and (iii) polarizing cytokines secreted by DCs (4). When presentation of antigen peptides by DCs occurs in the absence of costimulation, T cells become anergic (5). Anergy is a hyporesponsive state that retains T cells in an "off" mode under conditions in which immune activation is undesirable, as for the recognition of self-antigens and the maintenance of steady state. Understanding this process has become the focus of interest for the design of therapeutic strategies to silence autoreactive T cells in autoimmune diseases.

It has been reported that tolerogenic dendritic cells (tDCs) generated from monocytes of patients with multiple sclerosis (6), type 1 diabetes (T1D) (7), or rheumatoid arthritis (RA) (8) are able to induce a stable hyporesponsive state in $CD4^+$ T cells in an antigen-specific manner. In animal models of experimental autoimmune encephalomyelitis (EAE) (9) and collagen-induced arthritis (CIA) (10), inoculated tDCs induced antigen-specific T-cell anergy and thereby impeded disease progression. Furthermore, it has been reported that tDCs were capable of inducing donor-specific hyporesponsiveness and prolonging cardiac allograft survival in mouse models of transplantation (11, 12).

The current review takes a closer look at recent findings on T-cell anergy induced by tDCs and discusses the potential of T-cell anergy for clinical applications to control undesired immune responses mediated by $CD4^+$ T cells.

Tolerogenic Dendritic Cells and the Modulation of T-Cell Responses

Dendritic cells are professional antigen-presenting cells that are able to initiate and shape T-cell responses (13). Whether DCs induce T-cell immunity or tolerance is determined by their maturation state. Mature DCs are considered to be immunogenic as they display high levels of MHC-class II and costimulatory molecules on their surface (14) as well as a proinflammatory cytokine secretion profile (15), equipping them with the capacity to efficiently present antigen and provide activating signals to $CD4^+$ T cells, thus promoting their polarization toward T helper (Th) type 1, Th2, or Th17 cells. In contrast, immature DCs express low levels of MHC-II and costimulatory molecules and are mainly localized in blood and non-lymphoid tissues, where they act as sentinels specialized in capturing and recognizing antigens. A small proportion of DCs, termed semi-mature DCs, undergo partial maturation under steady-state conditions, resulting in upregulation of antigen presenting and lymph node homing capacity while proinflammatory cytokine secretion remains absent (16). Both immature and semi-mature DCs are regarded as tolerogenic because of their ability to favor T-cell differentiation to IL-10-secreting cells with regulatory properties (17). There are distinct mechanisms by which tDCs prevent T-cell responses against self-antigens *in vivo*, including deletion of autoreactive T cells, deviation of the T-cell cytokine secretion profile, generation of Tregs, and/or induction of anergy (1–3, 18). During the last decade, research has focused on the *in vitro* generation of tDCs with a stable phenotype. Human DCs are generated from peripheral blood monocytes cultured in the presence of GM-CSF and IL-4, and laboratory strategies to induce a tolerogenic phenotype include the addition of cytokines, such as IL-10 or TGF-β (19); pharmacological modulation by vitamin D3, rapamycin, or dexamethasone (20); or genetic modifications, such as IL-10 gene transduction; and silencing of CD40, CD80, or CD86 expression by RNA interference (21). Additional activation of tDCs by lipopolysaccharide (LPS) or its non-toxic analog monophosphoryl lipid A (MPLA) has been shown to improve their antigen-presenting capacity and to induce the expression of chemokine receptors that enable migration to secondary lymph nodes (22). Regardless of the strategy used for their generation, tDCs exhibit common characteristics such as low expression of costimulatory molecules, a decreased antigen-presenting capacity, and an anti-inflammatory cytokine secretion profile (20, 23, 24) and have been reported to inhibit the proliferation and activation of allogeneic and antigen-specific $CD4^+$ T cells (22), to promote the differentiation into IL-10-secreting Tregs (20, 25), and to render T cells anergic (19).

Additionally, generation of murine DCs from bone marrow (BMDCs) has been described using GM-CSF alone or in combination with IL-4 (26, 27) or Fms-like tyrosine kinase 3 (Flt3) (26). In a similar fashion of human DCs, a tolerogenic phenotype can be induced in murine DCs using different cytokines, pharmacological agents, or genetic modifications.

Recently, Helft and coworkers (2015) showed that the classical method to generate BMDCs using GM-CSF (28) produces heterogeneous $CD11c^+$ $MHCII^+$ populations that comprise conventional BMDCs, induced by GM-CSF (GM-DCs), and monocyte-derived macrophages, induced by GM-CSF (GM-Macs), that display distinct immune functions *in vitro* and *in vivo* (29). In the procedure of GM-DCs generation, many laboratories commonly employ magnetically enriched or FACS-sorted $CD11c^+$ assuming incorrectly that this DC population is homogeneous and that any cell-to-cell variation is the result of different maturation state (30, 31).

Despite this discovery, the modulatory effects of *ex vivo*-generated tolerogenic BMDCs, produced under GM-CSF protocol, have been extensively studied on $CD4^+$ T cells in murine models of autoimmune diseases and transplantation. These tDCs were shown to inhibit destructive immune responses in models of bone marrow and organ transplantation (32, 33) and to exert beneficial effects in mice with CIA (34, 35), diabetes (36), and EAE (37). Thus, although the classic mouse BMDCs generation protocol results in a heterogeneous population, their immune modulatory effects have been successfully demonstrated for a long time by several authors.

T-Cell Regulation or Anergy?

T-cell anergy is induced when negative signals outweigh the activatory signals provided by antigen-presenting cells. Originally, anergy was defined as unresponsive state induced in T cells that recognize antigen in the absence of costimulatory signals (38), usually provided by the binding of CD28 on T cells to its ligands, namely B7 molecules, expressed on DCs (39). Consequently, proliferation and cytokine production of T cells are impaired upon reencountering the same antigen (38). It has been observed that this hyporesponsive state could be reversed in the presence of IL-2 and that signaling through the IL-2 receptor prevented the establishment of anergy in the absence of costimulation, which is consistent with the *in vitro* definition of anergy (40). In contrast, the definition of *in vivo* anergy has been more difficult and presents characteristics that differ from *in vitro* induced anergy such as the failure of exogenous IL-2 to reverse the anergy state (41).

Anergy can also be induced by coinhibitory signals through CTLA-4 (cytotoxic T lymphocyte-associated protein 4) or PD-1 (programmed cell death 1) receptors (42–44). CTLA-4 interacts with B7 molecules, preferentially with CD80, while PD-1 binds to PD-L1 and PD-L2 ligands on DCs. Moreover, tissue-derived adenosine, acting via the adenosine A2A receptor (A2AR),

represents another important negative regulator of T-cell activation, able to promote long-term anergy even in the presence of costimulation (45).

Further studies show that anergy induction and maintenance depend on the presence of "anergy-associated factors" (2) such as GRAIL (gene related to anergy in lymphocytes), Cbl-b (Casitas B-cell lymphoma-b), and Itch (itchy homologue E3 ubiquitin protein ligase) (2), as well as the transcription factors Egr (early growth response) type 2 and 3 (46). GRAIL, CbI-b, and Itch are E3 ubiquitin ligases involved in cell signaling and protein ubiquitination and are modulated via the calcium/calcineurin pathway (47, 48).

Gene expression studies performed after TCR stimulation in the presence or absence of costimulation revealed upregulation of GRAIL in anergic $CD4^+$ T cells (49). The role of CbI-b was identified by comparing the proliferative response of peripheral T cells from Cbl-b knockout mice and wild-type mice. Peripheral T cells from Cbl-b knockout mice hyperproliferated (50), suggesting that loss of Cbl-b impairs the induction of a T-cell hyporesponsive state associated with tolerance (51). T cells from Itch-deficient mice were shown to be resistant to anergy induction, sustaining the role of Itch in the promotion of a hyporesponsive state (47).

Egr2 was demonstrated to be the major transcription factor for anergy induction both *in vitro* and *in vivo* (52), and its overexpression was shown to inhibit T-cell activation (46, 53). Egr2 and Egr3 direct the expression of anergy-inducing genes either in cooperation with the transcription factor NFAT (nuclear factor of activated T cells) (48) or in an independent manner. The proteins encoded by those Erg-regulated genes (e.g., Grail, Cbl-b, and Itch) are required to induce a functional unresponsiveness state through downregulation of TCR signaling by inactivation or degradation of signaling molecules (54).

It has been observed that some of the anergy-associated factors and pathways are also involved in the generation of Tregs (55) (**Table 1**). For example, GRAIL is up-regulated in $CD4^+CD25^+$ Tregs too, and its expression is linked to their regulatory activity (56). Cbl-b and Itch also regulate the development of $Foxp3^+$ Tregs in the periphery by modulating key components of TCR and TGF-β signaling pathways (57). Moreover, Egr2 is a central transcription factor for IL-10-secreting regulatory T cells expressing lymphocyte activation gene 3 (LAG-3) (58). Likewise, it has been reported that NFAT proteins are not only involved in the induction of T-cell anergy (59) but also mediate the suppressive function of Tregs by forming a cooperative complex with Foxp3 (60). Finally, A2AR signaling has been shown to induce T-cell anergy as well as $Foxp3^+$ and LAG-3^+ Tregs *in vivo* (45).

At the same time, it has been reported that anergic T cells can also acquire functions of Tregs. In an *in vivo* model of peripheral tolerance, antigen-specific anergic T cells were shown to secrete high levels of IL-10, suggesting that these anergic cells could act as Tregs (61). Steinbrink observed that anergic T cells, induced by IL-10-treated DCs, were able to suppress the activation and function of T cells in an antigen-specific manner (62). In this model, suppression was linked to CTLA-4-dependent cell cycle arrest (63). In another study, Pletinckx and colleagues showed that immature DCs were capable of converting anergic $CD4^+$ T cells into Foxp3-$IL10^+$ Tregs through engagement of CD28 and CTLA-4 (64).

T-cell anergy and Tregs induction are crucial mechanisms for the reestablishment of tolerance (9, 10, 65, 66), and although presenting different phenotypic and functional characteristics (**Table 1**), both mechanisms have in common the expression regulation of some genes, such as *Pd-1* (67, 68), *Icos* (55), *Lag3* (55), *Ctla-4* (55, 67), *Egr2* (55, 67), *Grail* (49, 56), *Cbl-b* (57), and *Itch* (57). Regarding the therapeutic potential of strategies inducing either anergy or Tregs, the question arises whether one or the other mechanism is more effective. Due to their capacity to efficiently suppress effector T-cell responses, Tregs were assumed to be the protagonists in tolerance induction. Deficiency or altered function of Tregs is associated with increased severity and activity of autoimmune disease (69). However, there is evidence that $Foxp3^+$ Tregs may convert into proinflammatory Th17 cells in a proinflammatory cytokine environment (70, 71). This plasticity or instability of Tregs is a disadvantage for their therapeutic application. On the other hand, the induction of a hyporesponsive state in T cells has proven to be stable and autoantigen-specific, enabling silencing of self-reactive T cells in autoimmune diseases (9, 10, 72–74). The possibility that these anergic T cells can acquire suppressive capacities would strengthen their therapeutic potential to control undesired immune responses.

TABLE 1 | Comparison of anergic T cells and regulatory T cells.

	Anergic T cells versus regulatory T cells	
Characteristic transcription factor	Egr2	Foxp3
Suppressor activity	Controversial	Yes
Cytokines	None/IL-10	IL-10, TGF-β
Proliferative responses	No	Yes
Role of costimulation	Absence required	Is required
Stability	Stable in the presence of the specific antigen	Stable; plasticity under certain conditions
Shared phenotype markers	GRAIL Cbl-b Itch CTLA-4 LAG-3	

Therapeutic Potential of T-Cell Anergy

The assumption that rendering autoreactive cells hyporesponsive might be a strategy to reestablish tolerance in conditions of autoimmunity and transplantation has prompted several preclinical studies to evaluate this approach in rodent models of multiple sclerosis, T1D, RA, and transplantation.

It has been previously reported that tDCs, modulated with vitamin D3 and loaded with myelin peptides, induce hyporesponsiveness of autologous myelin-specific T cells from multiple sclerosis patients *in vitro* (6). Mansilla and colleagues demonstrated the preventive and therapeutic effect of administering vitamin D3-modulated BMDCs stimulated with LPS and pulsed with encephalitogenic myelin oligodendrocyte glycoprotein (MOG) peptide *in vivo* in the EAE mouse model (9). Splenocytes from mice that received those tDCs showed reduced MOG-specific proliferation and increased IL-10 production. Another study by Zappia and coworkers reported that administration of

mesenchymal stem cells (MSCs), multipotent stromal cells with immunomodulatory properties, ameliorated EAE through the induction of T-cell hyporesponsiveness (72). In this model, MSCs inhibited the proliferative response of T cells from spleen and lymph nodes to MOG peptide and polyclonal stimuli, without increasing the frequency of Tregs. In accordance with previous reports, T-cell anergy was abrogated upon administration of IL-2 (31).

Regarding T1D, it has been shown that tDCs modulated with IL-10 and TGF-β and loaded with the pancreatic islet autoantigens insulin or glutamic acid decarboxylase 65 (GAD65) were able to induce antigen-specific hyporesponsiveness in $CD4^+$ T cells from patients *in vitro* (7). In a transfer model of T1D, administration of vitamin D3-treated DCs, loaded with the disease-relevant antigen BDC2.5 mimotope, induced antigen-specific hyporesponsiveness of autoreactive $CD4^+$ T cells *in vitro* and *in vivo* (73). Using a transgenic mouse model of T1D, based on the concomitant expression of influenza hemagglutinin (HA) in β cells of the pancreas (under control of the insulin promoter) and of an HA-specific MHC class II-restricted TCR, it has been demonstrated that myeloid-derived suppressor cells (MDSCs) pulsed with HA peptide, effectively suppressed HA-specific T-cell responses against pancreatic islet cells and thus prevented the development of diabetes. In this study, $Gr\text{-}1^+CD115^+$ MDSCs were obtained from syngeneic colon cancer MCA26 and from syngeneic lung carcinoma (74). The beneficial effect exerted by MDSCs involved the induction of T-cell hyporesponsiveness and the generation of Tregs.

Concerning RA, Harry and coworkers showed that tDCs from healthy donors and RA patients generated in the presence of dexamethasone, vitamin D3, and MPLA, and loaded with tuberculin purified protein derivative (PPD), induced only poor antigen-specific proliferation and production of IFN-γ and IL-17 by autologous T cells, even when T cells were previously primed by PPD-loaded mature DCs (8). In a mouse model of CIA, the same investigators showed that semi-mature BMDCs modulated with dexamethasone, vitamin D3, and LPS, and pulsed with the arthritogenic antigen collagen type II (CII), migrated to the inflamed articulation and reduced progression of arthritis (75). In this model, injection of those tDCs led to diminished CII-specific proliferation within splenocytes and decreased numbers of pathogenic Th17 cells while increasing the proportion of IL-10-producing $CD4^+$ T cells. In another study performed by Popov and coworkers, the administration of tDCs, modulated with the NF-κB inhibitor LF 15-0195 and pulsed with CII, delayed the onset of CIA and reduced the severity of the disease through the conversion of CII-specific T cells to a hyporesponsive state (10).

In a mouse model of transplantation, Fas ligand (FasL)-transfected murine BMDCs, displaying a tolerogenic phenotype, were able to inhibit allogeneic mixed leukocyte reaction *in vitro* and induced alloantigen-specific hyporesponsiveness *in vivo* dependent on FasL/Fas receptor interaction (11). The transfer of FasL-transfected tDCs significantly prolonged the survival of fully MHC-mismatched vascularized cardiac allografts by favoring the development of alloantigen-specific hyporesponsiveness (11). Another study demonstrated that dexamethasone-modulated and LPS-activated tDCs induce donor-specific T-cell hyporesponsiveness against the allograft and thereby prolong survival of cardiac allografts (12).

These *in vivo* studies support the suitability of strategies to induce antigen-specific T-cell anergy for the reestablishment of tolerance in patients with autoimmune disorders or transplants.

Currently, a number of clinical trials are being conducted. Giannoukakis et al. demonstrated safety of tDCs in T1D patients (76). In the study by Benham et al., tDCs from RA patients generated with BAY11-7082 and pulsed with citrullinated peptides showed a significant reduction of IL-6 response to vimentin in *ex vivo* antigen-specific T-cell proliferation assays. Effector T cells decreased after treatment and the underlying mechanism might include deletion or anergy in response to antigen recognition (77). Another study by Harry et al. is intended to assess safety, feasibility, and acceptability of Dex-VitD3-treated tDCs therapy (8). Additionally, the ability to modify antigen-specific pathogenic responses is also being evaluated using vaccines of synthetic peptides representing T-cell epitopes, such as Hsp90 on T1D patients (78), contributing to preservation of β-cell function and glycemic control, and dnaJP1 on RA patients (79), showing a reduction in the percentage of TNF-producing T cells.

Further mechanistic studies are needed in order to determine the efficacy of antigen-specific therapies for autoimmunity and the role of T-cell anergy.

Concluding Remarks

Suppression of antigen-specific T-cell responses either through the expansion of Tregs or the induction of anergy represents an attractive immunotherapeutic approach to target autoreactive T cells in autoimmune diseases. Despite the differences, both tolerance mechanisms share some fundamental signaling pathways and regulate the expression of common genes. The generation of Tregs has hitherto been the focus of interest; however, Tregs can exert unspecific regulation and may be prone to conversion into proinflammatory Th17 cells. In contrast, the induction of a stable hyporesponsive state appears to be a promising strategy to specifically silence self-reactive T cells in autoimmune diseases without undesired adverse effects. *In vitro* experiments confirmed that anergy induction efficiently prevents responses against disease-associated autoantigens in $CD4^+$ T cells of patients with autoimmune pathologies, including multiple sclerosis, T1D, or RA. *In vivo*, anergy induction in autoreactive $CD4^+$ T cells has been proven to control disease onset and progression in murine models of autoimmune diseases. The possibility that anergic T cells can also acquire suppressive capacities supports their fundamental role in the control of immune responses. Thus, T-cell anergy is an effective mechanism to eradicate aberrant T-cell responses to "self" and its induction by tDCs provides a promising therapeutic strategy for the reestablishment of self-tolerance in patients with autoimmune diseases.

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References

1. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol* (2010) **11**(1):7–13. doi:10.1038/ni.1818
2. Mueller D. Mechanisms maintaining peripheral tolerance. *Nat Immunol* (2010) **11**(1):21–7. doi:10.1038/ni.1817
3. Steinman R, Hawiger D, Nussenzweig M. Tolerogenic dendritic cells. *Annu Rev Immunol* (2003) **21**:685–711. doi:10.1146/annurev.immunol.21.120601.141040
4. van Gisbergen K, Paessens L, Geijtenbeek T, van Kooyk Y. Molecular mechanisms that set the stage for DC-T cell engagement. *Immunol Lett* (2005) **97**(2):199–208. doi:10.1016/j.imlet.2004.11.008
5. Schwartz R. Models of T cell anergy: is there a common molecular mechanism? *J Exp Med* (1996) **184**(1):1–8. doi:10.1084/jem.184.1.1
6. Raïch-Regué D, Grau-López L, Naranjo-Gómez M, Ramo-Tello C, Pujol-Borrell R, Martínez-Cáceres E, et al. Stable antigen-specific T-cell hyporesponsiveness induced by tolerogenic dendritic cells from multiple sclerosis patients. *Eur J Immunol* (2012) **42**(3):771–82. doi:10.1002/eji.201141835
7. Segovia-Gamboa N, Rodríguez-Arellano M, Rangel-Cruz R, Sánchez-Díaz M, Ramírez-Reyes J, Faradji R, et al. Tolerogenic dendritic cells induce antigen-specific hyporesponsiveness in insulin- and glutamic acid decarboxylase 65-autoreactive T lymphocytes from type 1 diabetic patients. *Clin Immunol* (2014) **154**(1):72–83. doi:10.1016/j.clim.2014.06.009
8. Harry R, Anderson A, Isaacs J, Hilkens C. Generation and characterisation of therapeutic tolerogenic dendritic cells for rheumatoid arthritis. *Ann Rheum Dis* (2010) **69**(11):2042–50. doi:10.1136/ard.2009.126383
9. Mansilla M, Sellès-Moreno C, Fàbregas-Puig S, Amoedo J, Navarro-Barriuso J, Teniente-Serra A, et al. Beneficial effect of tolerogenic dendritic cells pulsed with MOG autoantigen in experimental autoimmune encephalomyelitis. *CNS Neurosci Ther* (2015) **21**(3):222–30. doi:10.1111/cns.12342
10. Popov I, Li M, Zheng X, San H, Zhang X, Ichim T, et al. Preventing autoimmune arthritis using antigen-specific immature dendritic cells: a novel tolerogenic vaccine. *Arthritis Res Ther* (2006) **8**(5):R141. doi:10.1186/ar2031
11. Min W, Gorczynski R, Huang X, Kushida M, Kim P, Obataki M, et al. Dendritic cells genetically engineered to express Fas ligand induce donor-specific hyporesponsiveness and prolong allograft survival. *J Immunol* (2000) **164**(1):161–7. doi:10.4049/jimmunol.164.1.161
12. Emmer P, van der Vlag J, Adema G, Hilbrands L. Dendritic cells activated by lipopolysaccharide after dexamethasone treatment induce donor-specific allograft hyporesponsiveness. *Transplantation* (2006) **81**(10):1451–9. doi:10.1097/01.tp.0000208801.51222.bd
13. Lanzavecchia A, Sallusto F. Regulation of T cell immunity by dendritic cells. *Cell* (2001) **106**(3):263–6. doi:10.1016/S0092-8674(01)00455-X
14. Banchereau J, Steinman R. Dendritic cells and the control of immunity. *Nature* (1998) **392**(6673):245–52. doi:10.1038/32588
15. Hubo M, Trinschek B, Kryczanowsky F, Tuettenberg A, Steinbrink K, Jonuleit H. Costimulatory molecules on immunogenic versus tolerogenic human dendritic cells. *Front Immunol* (2013) **4**:82. doi:10.3389/fimmu.2013.00082
16. Lutz M. Therapeutic potential of semi-mature dendritic cells for tolerance induction. *Front Immunol* (2012) **3**:123. doi:10.3389/fimmu.2012.00123
17. Sallusto F, Lanzavecchia A. The instructive role of dendritic cells on T-cell responses. *Arthritis Res* (2002) **4**(Suppl 3):32. doi:10.1186/ar567
18. Rossetti M, Gregori S, Roncarolo M. Granulocyte-colony stimulating factor drives the in vitro differentiation of human dendritic cells that induce anergy in naïve T cells. *Eur J Immunol* (2010) **40**(11):3097–106. doi:10.1002/eji.201040659
19. Torres-Aguilar H, Aguilar-Ruiz S, González-Pérez G, Munguía R, Bajaña S, Meraz-Ríos M, et al. Tolerogenic dendritic cells generated with different immunosuppressive cytokines induce antigen-specific anergy and regulatory properties in memory CD4+ T cells. *J Immunol* (2010) **184**(4):1765–75. doi:10.4049/jimmunol.0902133
20. Anderson A, Sayers B, Haniffa M, Swan D, Diboll J, Wang X-N, et al. Differential regulation of naïve and memory CD4+ T cells by alternatively activated dendritic cells. *J Leukoc Biol* (2008) **84**(1):124–33. doi:10.1189/jlb.1107744
21. Zheng X, Suzuki M, Ichim T, Zhang X, Sun H, Zhu F, et al. Treatment of autoimmune arthritis using RNA interference-modulated dendritic cells. *J Immunol* (2010) **184**(11):6457–64. doi:10.4049/jimmunol.0901717
22. García-González P, Morales R, Hoyos L, Maggi J, Campos J, Pesce B, et al. A short protocol using dexamethasone and monophosphoryl lipid A generates tolerogenic dendritic cells that display a potent migratory capacity to lymphoid chemokines. *J Transl Med* (2013) **11**:128. doi:10.1186/1479-5876-11-128
23. Roncarolo M, Gregori S, Levings M. Type 1 T regulatory cells and their relationship with CD4+CD25+ T regulatory cells. *Novartis Found Symp* (2003) **252**:115. doi:10.1002/0470871628.ch9
24. Li D, Romain G, Flamar A-L, Duluc D, Dullaers M, Li X-H, et al. Targeting self- and foreign antigens to dendritic cells via DC-ASGPR generates IL-10-producing suppressive CD4+ T cells. *J Exp Med* (2012) **209**(1):109–21. doi:10.1084/jem.20110399
25. Unger W, Laban S, Kleijwegt F, van der Slik A, Roep B. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur J Immunol* (2009) **39**(11):3147–59. doi:10.1002/eji.200839103
26. Masurier C, Pioche-Durieu C, Colombo B, Lacave R, Lemoine F, Klatzmann D, et al. Immunophenotypical and functional heterogeneity of dendritic cells generated from murine bone marrow cultured with different cytokine combinations: implications for anti-tumoral cell therapy. *Immunology* (1999) **96**(4):569–77. doi:10.1046/j.1365-2567.1999.00728.x
27. Corsi M, Sandberg J, Wasserman K, Maes H, Kiessling R. Generation and function of bone marrow-derived dendritic cells from CD4/CD8(-/-) double-knockout mice. *Immunol Lett* (1999) **67**(3):243–9. doi:10.1016/S0165-2478(99)00018-8
28. Lutz M, Kukutsch N, Ogilvie A, Rössner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* (1999) **223**(1):77–92. doi:10.1016/s0022-1759(98)00204-x
29. Helft J, Böttcher J, Chakravarty P, Zelenay S, Huotari J, Schraml B, et al. GM-CSF mouse bone marrow cultures comprise a heterogeneous population of CD11c(+)MHCII(+) macrophages and dendritic cells. *Immunity* (2015) **42**(6):1197–211. doi:10.1016/j.immuni.2015.05.018
30. Shalek A, Satija R, Adiconis X, Gertner R, Gaublomme J, Raychowdhury R, et al. Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. *Nature* (2013) **498**(7453):236–40. doi:10.1038/nature12172
31. Shalek A, Satija R, Shuga J, Trombetta J, Gennert D, Lu D, et al. Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. *Nature* (2014) **510**(7505):363–9. doi:10.1038/nature13437
32. Lutz M, Suri R, Niimi M, Ogilvie A, Kukutsch N, Rössner S, et al. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. *Eur J Immunol* (2000) **30**(7):1813–22. doi:10.1002/1521-4141(200007)30:7<1813::AID-IMMU1813>3.0.CO;2-8
33. Giannoukakis N, Bonham C, Qian S, Chen Z, Peng L, Harnaha J, et al. Prolongation of cardiac allograft survival using dendritic cells treated with NF-kB decoy oligodeoxyribonucleotides. *Mol Ther* (2000) **1**(5 Pt 1):430–7. doi:10.1006/mthe.2000.0060
34. Healy L, Collins H, Thompson S. Systemic administration of tolerogenic dendritic cells ameliorates murine inflammatory arthritis. *Open Rheumatol J* (2008) **2**:71–80. doi:10.2174/1874312900802010071
35. van Duivenvoorde L, Han W, Bakker A, Louis-Plence P, Charbonnier L-M, Apparailly F, et al. Immunomodulatory dendritic cells inhibit Th1 responses and arthritis via different mechanisms. *J Immunol* (2007) **179**(3):1506–15. doi:10.4049/jimmunol.179.3.1506
36. Adorini L, Penna G, Giarratana N, Uskokovic M. Tolerogenic dendritic cells induced by vitamin D receptor ligands enhance regulatory T cells inhibiting allograft rejection and autoimmune diseases. *J Cell Biochem* (2003) **88**(2):227–33. doi:10.1002/jcb.10340
37. Chorny A, Gonzalez-Rey E, Fernandez-Martin A, Pozo D, Ganea D, Delgado M. Vasoactive intestinal peptide induces regulatory dendritic cells with therapeutic effects on autoimmune disorders. *Proc Natl Acad Sci U S A* (2005) **102**(38):13562–7. doi:10.1073/pnas.0504484102
38. Schwartz RH. T cell anergy. *Annu Rev Immunol* (2003) **21**:305–34. Epub 2002/12/10. doi:10.1146/annurev.immunol.21.120601.141110. PubMed
39. Bour-Jordan H, Esensten J, Martinez-Llordella M, Penaranda C, Stumpf M, Bluestone J. Intrinsic and extrinsic control of peripheral T-cell tolerance by costimulatory molecules of the CD28/B7 family. *Immunol Rev* (2011) **241**(1):180–205. doi:10.1111/j.1600-065X.2011.01011.x

40. Boussiotis V, Barber D, Nakarai T, Freeman G, Gribben J, Bernstein G, et al. Prevention of T cell anergy by signaling through the gamma c chain of the IL-2 receptor. *Science* (1994) **266**(5187):1039–42. doi:10.1126/science.7973657
41. Chiodetti L, Choi S, Barber D, Schwartz R. Adaptive tolerance and clonal anergy are distinct biochemical states. *J Immunol* (2006) **176**(4):2279–91. doi:10.4049/jimmunol.176.4.2279
42. Fife B, Bluestone J. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunol Rev* (2008) **224**:166–82. doi:10.1111/j.1600-065X.2008.00662.x
43. Greenwald R, Boussiotis V, Lorsbach R, Abbas A, Sharpe A. CTLA-4 regulates induction of anergy in vivo. *Immunity* (2001) **14**(2):145–55. doi:10.1016/S1074-7613(01)00097-8
44. Bishop K, Harris J, Mordes J, Greiner D, Rossini A, Czech M, et al. Depletion of the programmed death-1 receptor completely reverses established clonal anergy in CD4(+) T lymphocytes via an interleukin-2-dependent mechanism. *Cell Immunol* (2009) **256**(1–2):86–91. doi:10.1016/j.cellimm.2009.01.008
45. Zarek P, Huang C-T, Lutz E, Kowalski J, Horton M, Linden J, et al. A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. *Blood* (2008) **111**(1):251–9. doi:10.1182/blood-2007-03-081646
46. Safford M, Collins S, Lutz M, Allen A, Huang C-T, Kowalski J, et al. Egr-2 and Egr-3 are negative regulators of T cell activation. *Nat Immunol* (2005) **6**(5):472–80. doi:10.1038/ni1193
47. Heissmeyer V, Macián F, Im S-H, Varma R, Feske S, Venuprasad K, et al. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat Immunol* (2004) **5**(3):255–65. doi:10.1038/ni1047
48. Macián F, García-Cózar F, Im S-H, Horton H, Byrne M, Rao A. Transcriptional mechanisms underlying lymphocyte tolerance. *Cell* (2002) **109**(6):719–31. doi:10.1016/S0092-8674(02)00767-5
49. Anandasabapathy N, Ford G, Bloom D, Holness C, Paragas V, Seroogy C, et al. GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells. *Immunity* (2003) **18**(4):535–47. doi:10.1016/s1074-7613(03)00084-0
50. Bachmaier K, Krawczyk C, Kozieradzki I, Kong Y, Sasaki T, Oliveira-dos-Santos A, et al. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* (2000) **403**(6766):211–6. doi:10.1038/35003228
51. Jeon M-S, Atfield A, Venuprasad K, Krawczyk C, Sarao R, Elly C, et al. Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction. *Immunity* (2004) **21**(2):167–77. doi:10.1016/j.immuni.2004.07.013
52. Zheng Y, Zha Y, Driessens G, Locke F, Gajewski T. Transcriptional regulator early growth response gene 2 (Egr2) is required for T cell anergy in vitro and in vivo. *J Exp Med* (2012) **209**(12):2157–63. doi:10.1084/jem.20120342
53. Harris J, Bishop K, Phillips N, Mordes J, Greiner D, Rossini A, et al. Early growth response gene-2, a zinc-finger transcription factor, is required for full induction of clonal anergy in CD4+ T cells. *J Immunol* (2004) **173**(12):7331–8. doi:10.4049/jimmunol.173.12.7331
54. Mueller D. E3 ubiquitin ligases as T cell anergy factors. *Nat Immunol* (2004) **5**(9):883–90. doi:10.1038/ni1106
55. Knoechel B, Lohr J, Zhu S, Wong L, Hu D, Ausubel L, et al. Functional and molecular comparison of anergic and regulatory T lymphocytes. *J Immunol* (2006) **176**(11):6473–83. doi:10.4049/jimmunol.176.11.6473
56. MacKenzie D, Schartner J, Lin J, Timmel A, Jennens-Clough M, Fathman C, et al. GRAIL is up-regulated in CD4+ CD25+ T regulatory cells and is sufficient for conversion of T cells to a regulatory phenotype. *J Biol Chem* (2007) **282**(13):9696–702. doi:10.1074/jbc.M604192200
57. Venuprasad K. Cbl-b and itch: key regulators of peripheral T-cell tolerance. *Cancer Res* (2010) **70**(8):3009–12. doi:10.1158/0008-5472.can-09-4076
58. Okamura T, Fujio K, Shibuya M, Sumitomo S, Shoda H, Sakaguchi S, et al. CD4+CD25-LAG3+ regulatory T cells controlled by the transcription factor Egr-2. *Proc Natl Acad Sci U S A* (2009) **106**(33):13974–9. doi:10.1073/pnas.0906872106
59. Bandyopadhyay S, Soto-Nieves N, Macián F. Transcriptional regulation of T cell tolerance. *Semin Immunol* (2007) **19**(3):180–7. doi:10.1016/j.smim.2007.02.006
60. Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan A, Stroud J, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* (2006) **126**(2):375–87. doi:10.1016/j.cell.2006.05.042
61. Buer J, Lanoue A, Franzke A, Garcia C, von Boehmer H, Sarukhan A. Interleukin 10 secretion and impaired effector function of major histocompatibility complex class II-restricted T cells anergized in vivo. *J Exp Med* (1998) **187**(2):177–83. doi:10.1084/jem.187.2.177
62. Steinbrink K. CD4+ and CD8+ anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* (2002) **99**(7):2468–76. doi:10.1182/blood.V99.7.2468
63. Kubsch S, Graulich E, Knop J, Steinbrink K. Suppressor activity of anergic T cells induced by IL-10-treated human dendritic cells: association with IL-2- and CTLA-4-dependent G1 arrest of the cell cycle regulated by p27Kip1. *Eur J Immunol* (2003) **33**(7):1988–97. doi:10.1002/eji.200323600
64. Pletinckx K, Vaeth M, Schneider T, Beyersdorf N, Hünig T, Berberich-Siebelt F, et al. Immature dendritic cells convert anergic nonregulatory T cells into Foxp3(-) IL-10(+) regulatory T cells by engaging CD28 and CTLA-4. *Eur J Immunol* (2015) **45**(2):480–91. doi:10.1002/eji.201444991
65. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* (2007) **110**(4):1225–32. doi:10.1182/blood-2006-12-064527
66. Sakaguchi S, Wing K, Miyara M. Regulatory T cells – a brief history and perspective. *Eur J Immunol* (2007) **37**(Suppl 1):23. doi:10.1002/eji.200737593
67. Lechner O, Lauber J, Franzke A, Sarukhan A, von Boehmer H, Buer J. Fingerprints of anergic T cells. *Curr Biol* (2001) **11**(8):587–95. doi:10.1016/S0960-9822(01)00160-9
68. Gavin M, Clarke S, Negrou E, Gallegos A, Rudensky A. Homeostasis and anergy of CD4(+)CD25(+) suppressor T cells in vivo. *Nat Immunol* (2002) **3**(1):33–41. doi:10.1038/ni743
69. Dejaco C, Duftner C, Grubeck-Loebenstein B, Schirmer M. Imbalance of regulatory T cells in human autoimmune diseases. *Immunology* (2006) **117**(3):289–300. doi:10.1111/j.1365-2567.2005.02317.x
70. Yang X, Nurieva R, Martinez G, Kang H, Chung Y, Pappu B, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* (2008) **29**(1):44–56. doi:10.1016/j.immuni.2008.05.007
71. Osorio F, LeibundGut-Landmann S, Lochner M, Lahl K, Sparwasser T, Eberl G, et al. DC activated via dectin-1 convert Treg into IL-17 producers. *Eur J Immunol* (2008) **38**(12):3274–81. doi:10.1002/eji.200838950
72. Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, et al. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* (2005) **106**(5):1755–61. doi:10.1182/blood-2005-04-1496
73. Ferreira GB, Gysemans CA, Demengeot J, da Cunha JP, Vanherwegen AS, Overbergh L, et al. 1,25-Dihydroxyvitamin D3 promotes tolerogenic dendritic cells with functional migratory properties in NOD mice. *J Immunol* (2014) **192**(9):4210–20. doi:10.4049/jimmunol.1302350
74. Yin B, Ma G, Yen C-Y, Zhou Z, Wang GX, Divino CM, et al. Myeloid-derived suppressor cells prevent type 1 diabetes in murine models. *J Immunol* (2010) **185**(10):5828–34. doi:10.4049/jimmunol.0903636
75. Stoop JN, Harry RA, von Delwig A, Isaacs JD, Robinson JH, Hilkens CM. Therapeutic effect of tolerogenic dendritic cells in established collagen-induced arthritis is associated with a reduction in Th17 responses. *Arthritis Rheum* (2010) **62**(12):3656–65. doi:10.1002/art.27756
76. Giannoukakis N, Phillips B, Finegold D, Harnaha J, Trucco M. Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. *Diabetes Care* (2011) **34**(9):2026–32. doi:10.2337/dc11-0472
77. Benham H, Nel H, Law S, Mehdi A, Street S, Ramnoruth N, et al. Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype-positive rheumatoid arthritis patients. *Sci Transl Med* (2015) **7**(290):290ra87. doi:10.1126/scitranslmed.aaa9301
78. Raz I, Ziegler A, Linn T, Schernthaner G, Bonnici F, Distiller L, et al. Treatment of recent-onset type 1 diabetic patients with DiaPep277: results of a double-blind, placebo-controlled, randomized phase 3 trial. *Diabetes Care* (2014) **37**(5):1392–400. doi:10.2337/dc13-1391
79. Koffeman E, Genovese M, Amox D, Keogh E, Santana E, Matteson E, et al. Epitope-specific immunotherapy of rheumatoid arthritis: clinical responsiveness occurs with immune deviation and relies on the expression of a cluster of

molecules associated with T cell tolerance in a double-blind, placebo-controlled, pilot phase II trial. *Arthritis Rheum* (2009) **60**(11):3207–16. doi:10.1002/art.24916

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Alloreactive Regulatory T Cells Allow the Generation of Mixed Chimerism and Transplant Tolerance

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The induction of donor-specific transplant tolerance is one of the main goals of modern immunology. Establishment of a mixed chimerism state in the transplant recipient has proven to be a suitable strategy for the induction of long-term allograft tolerance; however, current experimental recipient preconditioning protocols have many side effects, and are not feasible for use in future therapies. In order to improve the current mixed chimerism induction protocols, we developed a non-myeloablative bone-marrow transplant (NM-BMT) protocol using retinoic acid (RA)-induced alloantigen-specific Tregs, clinically available immunosuppressive drugs, and lower doses of irradiation. We demonstrate that RA-induced alloantigen-specific Tregs in addition to a NM-BMT protocol generates stable mixed chimerism and induces tolerance to allogeneic secondary skin allografts in mice. Therefore, the establishment of mixed chimerism through the use of donor-specific Tregs rather than non-specific immunosuppression could have a potential use in organ transplantation.

Keywords: alloreactive regulatory T cells, mixed chimerism, transplant tolerance, organ transplantation, non-myeloablative conditioning

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INTRODUCTION

Achieving transplant tolerance as a means to improve the outcome of organ transplantation is one of the main challenges of modern immunology. Current immunosuppressive drug regimens succeed in preventing acute organ rejection; however, they fail to induce long-term operational tolerance and cannot prevent chronic rejection (1–3).

The generation of mixed chimerism has become an attractive alternative to achieving the induction of long-term tolerance to allografts (4, 5). Mixed chimerism is defined as the coexistence of donor and recipient hematopoietic cell precursors in a pre-conditioned allogeneic bone-marrow transplant (BMT) recipient (4–6). There is robust evidence showing that the induction of mixed chimerism allows the generation of alloantigen-specific tolerance in animal models (7–11) and humans (12–15).

Abbreviations: APCs, antigen-presenting cells; BMT, bone-marrow transplant; NM-BMT, non-myeloablative bone-marrow transplantation; RA, retinoic acid; RA-iTregs, retinoic acid-inducible regulatory T cells; TBI, total body irradiation; Tregs, regulatory T cells.

However, the generation of mixed chimerism requires myeloablative preconditioning protocols that can be risky and ethically unfeasible for application on patients who are eligible for a transplant. Different types of regimens have been designed to reduce the myeloablation necessary for the establishment of mixed chimerism (16, 17). Many of them use non-myeloablative doses of total body irradiation (TBI) (18, 19), either alone or in combination with the administration of immunosuppressive drugs (20), depleting antibodies (21–23), and/or co-stimulation blockers (7, 24). The inhibition of CD40/CD40L pathway is critical to induce mixed chimerism without T cell depletion (7, 25, 26). Nevertheless, CD40/CD40L pathway inhibition has been associated with thromboembolic complications (27, 28), and its approval for clinical use remains doubtful.

The use of cellular therapy, which mediates immune tolerance in an antigen-specific manner, may reduce the degree of immunosuppression or co-stimulation blockade necessary for the establishment of mixed chimerism to a minimum. Regulatory T cells have been widely studied for their potential use in this type of therapies. These cells prevent autoimmune and inflammatory diseases, regulate immune responses against viral, bacterial, or parasitic infections, and can also restrain responses directed toward tumors or transplanted tissues (29–33). Two different types of Tregs have been described; natural Tregs CD4 + CD25 + Foxp3 + (nTregs), which are generated in the thymus, and induced Tregs CD4 + CD25 + Foxp3 + (iTregs), which develop in the periphery from naïve CD4 + T cells. Both subsets of Tregs mediate their suppression in an antigen-specific manner (34).

Regulatory T cells have been used in several different cell therapies to treat experimental pathological models. These cells have been shown to prevent graft-versus-host disease (35), delay allograft rejection (36–38), and also provide protection for autoimmune disease therapies (39). Most of the protocols expand the existing pool of Treg cells, through the use of CD3 + CD28+ beads to obtain sufficient number of Treg to use in cellular therapy (40, 41). However, the polyclonal expansion of Tregs could generate a pool of cells with a wide spectrum of specificities.

Our group (41, 42) and others (35) have previously demonstrated the role of retinoic acid (RA) in enhancing the differentiation of CD4 + T cell to iTregs (42, 44–46). We developed a protocol to generate iTregs from naïve T cells in presence of allogeneic CD11c-enriched antigen-presenting cells (APCs), RA, and TGF-β. These, allogeneic RA-iTregs express several immunosuppressive molecules on the surface, have a potent suppressive capacity, show a stable phenotype, and avoid alloantigen-specific skin transplant rejection (42).

In this study, we sought to determine whether allogeneic RA-iTregs could improve current preconditioning protocols of BMT to establish mixed chimerism and induce donor-specific tolerance (25, 43). To test this, we generated a new non-myeloablative bone-marrow transplant (NM-BMT) protocol using allogeneic RA-iTregs, together with Rapamycin, Abatacept (CTLA4Ig), combined with low-dose TBI. This protocol allows the generation of stable mixed chimerism that induces tolerance to allogeneic skin grafts without the administration of additional immunosuppressive drugs.

MATERIALS AND METHODS

Animals

Six to 12-week-old C57BL/6 (Donor, H-2b), BALB/c, and B10.BR (Recipients, H-2d and H-2k, respectively) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were kept in an animal facility under standard housing guidelines. The animal work was carried out under the institutional regulation of Fundacion Ciencia & Vida and was locally approved by an ethical review committee.

Antibodies and Reagents

The characterization of Tregs and the analysis of different populations in blood from NM-BMT mice were performed by flow cytometry using the following antibodies: anti-H2K^b FITC (AF6.88.6), anti-H2K^d PE (SF1-1.1), anti GITR FITC (DTA-1), anti-CD19 APC-H7 (1D3), and anti-CD25 FITC (7D4) from BD Pharmingen (Franklin Lakes, NJ, USA); anti-CD4 FITC (RM4-5), anti-CD4 PE-Cy5 (RM4-5), anti-CD25 PE (PC61.5), anti-CD25 APC (PC61, 5), anti-CD8a PE-Cy7 (53-6.7), anti-GR1 PE-Cy7 (GL7), anti-FoxP3 PE-Cy7 (FJK-16s), anti-CD4 APC (6K1.5), anti-CD3 APC (17A2), and anti-CD16/32 (2.4G2) from eBioscience (San Diego, CA, USA); and anti-CD4 PE (RM4-5) and anti-F4/80 APC-Cy7 (BM8) from BioLegend (San Diego, CA, USA).

Recombinant human TGF-β1 was purchased from eBioscience (San Diego, CA, USA), mouse recombinant IL-2 from R&D System (Minneapolis, MN, USA), and RA from Sigma-Aldrich (St Louis, MO, USA).

Non-Myeloablative Bone-Marrow Transplant

BALB/c mice were irradiated with high-energy photons using the Oncor Impression PlusLinear Accelerator (Siemens, Munich, Germany). Mice received 3 Gy of TBI 1 day before BMT (day −1).

Groups of age-matched recipient mice received three doses of Rapamycin (LC Laboratories, Woburn, MA, USA) (5 mg/kg) at day −1, 0, and 2 (25). At day 0, BALB/c mice received a dose of 20×10^6 bone-marrow (BM) cells from C57BL/6 mice with or without 2.2×10^6 of RA-iTregs generated from BALB/c mice. On day 2, recipient mice received a single dose (24 mg/kg) of Abatacept (CTLA4Ig) (Orencia, Bristol-Myers Squibb Pharmaceuticals, Princeton, NJ, USA).

Generation and Isolation of Retinoic Acid-Induced Regulatory T Cells

Alloantigen-specific RA-iTregs were generated following published protocols (43, 44). Briefly, RA-iTregs were generated by stimulating naive T cells from BALB/c mice (sorted as CD4 + CD25−CD62LhiCD44low) in the presence of C57BL/6 APCs plus RA (10 nM), TGF-β (2 ng/ml), and IL-2 (10 ng/ml). After 6 days of culture, RA-iTregs were further purified by cell sorter as CD4+ CD25hi cells using a FACSAria II Cell Sorter (BD Bioscience, San Diego, CA, USA). The purity of the RA-iTregs preparation was always over 95%. Polyclonal RA-iTregs were generated by stimulating naive T cells from BALB/c mice in the presence of BALB/c APCs plus anti-CD3 mAb RA (10 nM), TGF-beta (2 ng/ml), and IL-2 (10 ng/ml).

The phenotype of RA-iTreg was determined by flow cytometry (FACS Canto II, BD Bioscience, San Diego, CA, USA). The analysis was carried out using the FlowJo version 8.7 software (Tree Star Inc. Ashland, OR, USA). Intracellular Foxp3 staining was done using the Foxp3 staining Kit (eBioscience, San Diego, CA, USA) following the manufacturer's instructions.

Skin Transplantation

For skin transplant experiments, we used C57BL/6 (H-2^b) mice as skin donors and BALB/c (H-2^d) mice as transplant recipients. Skin transplantation was performed 4 weeks post BM transplant. The recipient mice were anesthetized with 3% XEVORane (Abbot, Buenos Aires, IL, Argentina). Full thickness tail skin from mismatched C57BL/6 mice of ~1 cm × 1 cm was transplanted on the left lateral flank of recipient mice. Mice were bandaged for 10 days, and the acceptance or rejection of the graft was analyzed by macroscopic visual inspections at short time intervals. Rejection was determined when transplanted skin formed a dry scar (44, 45, 49, 50).

Chimerism Analysis by Flow Cytometry

To evaluate mixed chimerism, we measured the presence of donor MHC-I positive cells in blood and expressed it as the percentage of donor MHC-I positive cells over total leukocyte lineage. The percentages of the different donor populations were determined by labeling the cells with anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-GR1, and anti-F4/80 in combination with donor MHC-I antibody. We performed the analyses on 3, 4, 7, 10, 13, and 16 weeks after NM-BMT.

ELISPOT Assays

Splenocytes were recovered from BALB/c recipients 110 to 120 days after skin graft transplantation. These cells were re-stimulated for 24 h with previously irradiated (30 Gy) allogeneic (C57BL/6) or syngeneic (BALB/c) splenocytes. To measure the frequency of IFN-γ, IL-4-, and IL-17-producing cells, we used the mouse ELISPOT Ready-SET-go Kit (eBioscience San Diego, CA, USA) according to the manufacturer's instructions. The frequencies of cytokine-secreting cells were expressed as the number of cytokine-producing cells per 1×10^5 responder cells. All assays were done in triplicate.

In vitro MLR Suppression Assay

CD4+CD25− responder T cells (5×10^4) were sorted from BALB/c mice and labeled with CellTrace Violet dye (Life Technologies). As stimulator cells, we used 1×10^5 previously irradiated splenocytes (30 Gy) from C57BL/6 (allogeneic), B10.BR (third-party) or BALB/c (syngeneic) mice. The responder and stimulator cells were co-cultured at different ratios with CMTMR-labeled RA-iTreg or with anti-CD3 mAb. After 5 days, CellTrace Violet dilution in responder cells was analyzed by flow cytometry.

Statistical Analysis

Statistical analyzes were performed using Prism program for Macintosh, version 4.0b (GraphPad Software Inc.). In order to establish the statistical distribution of the results, we used the Shapiro–Wilk normality test. Depending on the sampling distribution, a non-parametric Mann–Whitney test, a parametric Student's *t*-test, or one-way ANOVA was used. The graft survival curves are shown using a Kaplan–Meier analysis and the statistical significance was estimated by LogRank test. *p*-values ≤ 0.05 were considered statically significant. The data are presented as mean ± error standard (SEM).

RESULTS

Donor-Specific RA-iTreg Together with a NM-BMT Protocol Induces Stable Mixed Chimerism

In a previous study, we generated donor-specific retinoic acid-induced Tregs (RA-iTregs) and used these cells to prevent skin allograft rejection in an immunodeficient Rag1−/− mouse model (42). However, it remained to be established whether these donor-specific RA-iTregs were also able to generate mixed chimerism and allow transplantation tolerance in immunocompetent mice. To test this, we designed a NM-BMT protocol that may be translated to clinical use. This NM-BMT protocol consists of 3 Gy TBI (at day −1), three doses of Rapamycin (5 mg/kg at day −1, 0, and 2), and a single dose of Abatacept (CTLA4Ig) (24 mg/kg at day +2). Additionally, some of the recipient mice received a single dose of RA-iTregs at day 0 (see diagram on **Figure 1A**).

Next, we sought to study the ability of allospecific Tregs to induce mixed chimerism. For this, we stimulated naive T cells obtained from BALB/c mice (H-2^d) with allogeneic APCs from C57BL/6 mice (H-2^b) in the presence of TGF-β, RA, and IL-2. Figure S1A in Supplementary Material shows that RA-iTregs generated after 6 days of culture express several markers related to Treg function such as CTLA4, LAG3, CD73, CD39, GARP, CD103, and GITR. Then, we tested whether these RA-iTregs could specifically inhibit the allogeneic response. As shown in Figure S1B in Supplementary Material, RA-iTregs specifically inhibited the proliferation of T cells cultured with C57BL/6 APCs but not cultures done with third-party APCs (B10.BR). Moreover, allogeneic RA-iTregs are more efficient than polyclonal RA-iTregs at inhibiting the proliferation of T cells in mixed leukocyte reactions (Figure S2 in Supplementary Material).

To study the ability of these allospecific RA-iTregs in promoting mixed chimerism, donor H-$2K^b$ cells in peripheral blood samples of NM-BMT mice were assessed by flow cytometry. As shown in **Figure 1B**, mice receiving RA-iTregs presented donor-derived (H-$2K^{b+}$) cells compared with the group that did not receive RA-iTregs. This result demonstrates that RA-iTregs can generate mixed chimerism in our NM-BMT protocol.

To evaluate the stability of mixed chimerism generated by a single dose of RA-iTregs, we determined over time the presence of H-$2K^b$ cells in peripheral blood of mice. **Figure 1C** shows that the group that did not receive RA-iTregs presented lower levels of chimerism, disappearing by 7 weeks after BMT. By contrast, mice treated with RA-iTregs displayed a statistically significant percentage of mixed chimerism up to 16 weeks post BMT, indicating that a single dose of RA-iTregs is sufficient to induce a stable mixed chimerism.

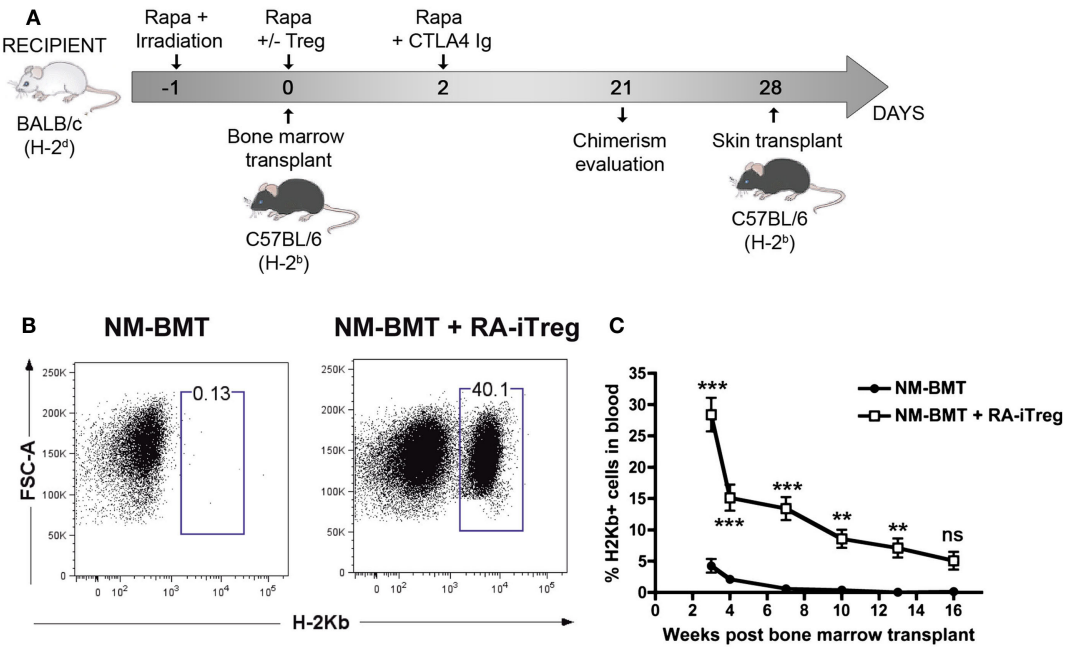

FIGURE 1 | Alloantigen-specific RA-iTregs allow the generation of stable mixed chimerism in NM-BMT mice. **(A)** Schematic representation of NM-BMT regimen. BALB/c mice received NM-BMT regimens that consisted in the administration of low doses of irradiation (3 Gy of TBI at day −1), together with co-stimulation blockade with Abatacept (CTLA4Ig) (24 mg/kg at day 2), and three doses of Rapamycin (5 mg/kg at days −1, 0, and 2). At day 0, mice received 20×10^6 bone-marrow cells from C57BL/6 mice and 2.2×10^6 BALB/c RA-iTreg cells or PBS. **(B)** Chimerism at 3 weeks post-transplantation was analyzed by the presence of H-2K^b positive cells in blood. **(C)** Kinetics of mixed chimerism, analyzed as the presence of H-2K^b positive cells in peripheral blood samples from NM-BMT mice treated with (▫) or without (•) RA-iTregs. The statistical one-way ANOVA test was used. Bars represent the SE. The data represent seven independent experiments. Rapa, Rapamycin; Aba, Abatacept; RA-iTregs, retinoic acid-induced Tregs cells; BMT: Bone-marrow transplantation. ***$p < 0.0001$; **$p < 0.001$; *$p < 0.01$.

Next, we evaluated reconstitution of the chimera with multi-lineage donor leukocytes. For this, we analyzed CD4+ and CD8+ T cell subsets, B lymphocytes (CD19+), monocytes (F4/80+), and granulocytes (GR-1+) by flow cytometry in blood samples at different time points post NM-BMT (Figures S3A,B in Supplementary Material). We observed increasing percentages of T lymphocytes, with a similar contribution from CD4+ and CD8+ T cells. The levels of B cells were maintained over time and represented a substantial percentage of total chimerism. Furthermore, we observed granulocyte and monocyte reconstitution. However, in contrast to lymphoid cells, myeloid cells showed a decrease over time. Therefore, we observed that the mice subjected to NM-BMT in combination with RA-iTregs repopulated all cellular hematopoietic lineages from donor precursors.

RA-iTregs Together with NM-BMT Prolong Allograft Survival

To determine whether chimeras obtained with RA-iTregs induce tolerance mechanisms in BALB/c recipient mice, we performed allogeneic skin grafts using C57BL/6 mice as skin donors. Transplants were done 4 weeks after the initiation of the non-myeloablative protocol. We separated recipient mice into three groups: non-treated mice, NM-BMT mice, and RA-iTreg treated NM-BMT mice. **Figure 2A** shows the presence of skin and black hair at the site of the transplant 100 days after skin transplant in RA-iTreg-treated mice. Mice that received only NM-BMT quickly rejected the allograft as shown by the scar at the site of operation. **Figure 2B** shows that Treg-treated NM-BMT mice showed a significant increase ($p < 0.0001$) in the survival of the transplanted skin, exceeding 100 days post-transplant, without the need for any additional immunosuppression. Therefore, mixed chimeras generated with the combination of NM-BMT and RA-iTregs induce tolerance mechanisms that allow the acceptance of allogeneic skin grafts.

RA-iTregs Together with NM-BMT Inhibit Allogeneic-Induced Th1 Cellular Responses

The main factors involved in organ rejection are cellular immune responses mediated by Th1 and Th17 cells (46, 47). It has been reported that the induction of mixed chimerism promotes Th2 responses that favor alloimmune tolerance (53). Thus, we investigated by ELISPOT assays the type of alloimmune response produced by RA-iTregs-treated NM-BMT mice at the end of the skin transplant protocol. For this, splenocytes from transplanted mice were co-cultured for 24 h with syngeneic or allogeneic splenocytes on pre-coated ELISPOT plates to analyze IFN-γ-, IL-4, and IL-17 secreting cells. We used as controls splenocytes from NM-BMT mice that rejected the skin transplant. As shown in **Figure 3**, we observed a strong Th1 response, as measured by IFN-γ secretion,

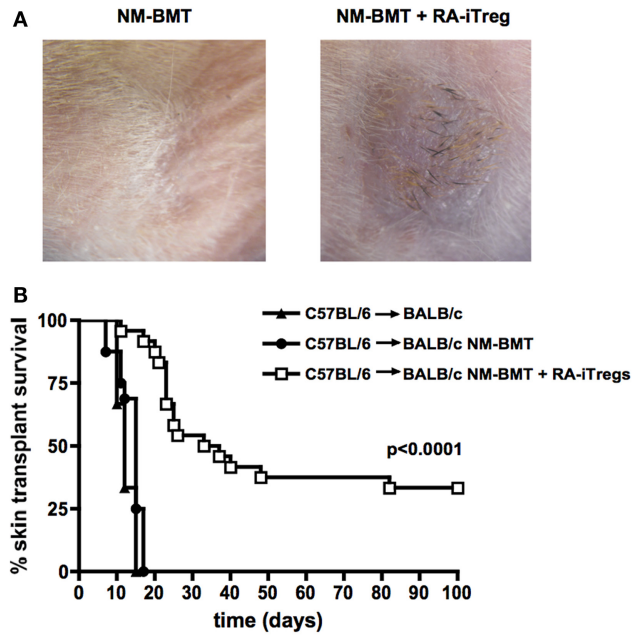

FIGURE 2 | NM-BMT mice treated with RA-iTregs have a prolonged allogeneic skin graft survival. Skin transplants were performed using C57BL/6 mice as donors of tail skin in untreated BALB/c mice (BALB/c) (▲) (n = 3); bone-marrow-transplanted BALB/c mice (BALB/c NM-BMT) (●) (n = 13); or bone-marrow-transplanted BALB/c plus RA-iTregs (BALB/c NM-BMT + RA-iTreg) (□) (n = 17) as receptors. **(A)** Image of skin transplant at day 50 post-transplant. **(B)** Kaplan–Meier survival analysis of skin allograft transplantation for 100 days post-transplant. LogRank Statistical test (comparison of survival curves) was performed. p-value <0.05. The results represent four independent experiments.

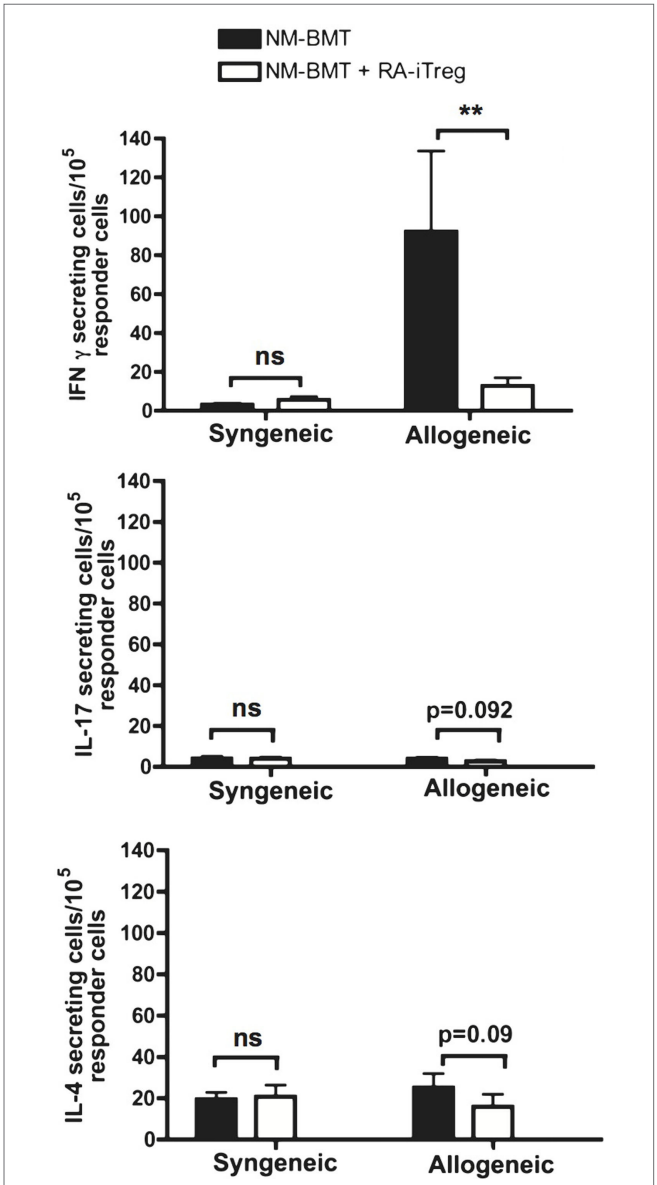

FIGURE 3 | Th1 allogeneic immune response is inhibited in NM-BMT mice that received RA-iTregs. One hundred days after skin transplant, splenocytes from NM-BMT mice treated with or without RA-iTregs were co-cultured with APC from BALB/c (syngeneic) or C57BL/6 (allogeneic) mice. IFN-γ, IL-17, and IL-4 cytokine secretion was measured by ELISPOT. Bars show the SE. The Mann–Whitney–Wilcoxon statistical test for non-parametric distributions was used. The results represent two independent experiments.

only when splenocytes from NM-BMT are stimulated with allogeneic splenocytes. We observed that RA-iTregs-treated NM-BMT had a significant decrease in IFN-γ secreting cells comparable to the level found in syngeneic cocultures ($p = 0.008$). We did not see any significant IL-4 or Il-17 cytokine secretion in all conditions, indicating that Th2 and Th17 cellular responses do not mediate allograft rejection in our setting. These results suggest that peripheral tolerance mechanisms induced in RA-iTreg-treated NM-BMT mice effectively abolish Th1 alloimmune responses.

Chimeric Mice Exhibit Donor-Derived Regulatory T Cells

To study the mechanism of tolerance induction in RA-iTreg-treated NM-BMT mice, we evaluated the presence of CD4 + CD25 + FoxP3+ Tregs derived from donor precursors cells (H-2^b) in these mice. As shown in **Figure 4**, 100 days after skin transplant, we found donor-derived H-2^b + CD4 + CD25 + FoxP3+ Tregs in blood and spleen of NM-BMT mice treated with RA-iTregs. When we correlated the presence of donor Tregs with skin transplant acceptance or rejection (**Figure 4B**), we observed that some mice that accepted allogeneic skin transplant presented donor Tregs in spleen and blood. However, we could not detect donor Tregs in blood and spleen in mice that rejected allogeneic skin.

DISCUSSION

In this work, we developed a protocol using RA-iTregs to induce mixed chimerism. Here, we combine RA-iTregs with a NM-BMT avoiding the need of CD40/CD40L signaling inhibition. This NM-BMT protocol consisted of a low dose of TBI (3 Gy), three doses of the immunosuppressive drug Rapamycin, and one dose of co-stimulation blockade using Abatacept (CTLA4Ig). We demonstrate that the use of RA-iTregs with this non-myeloablative

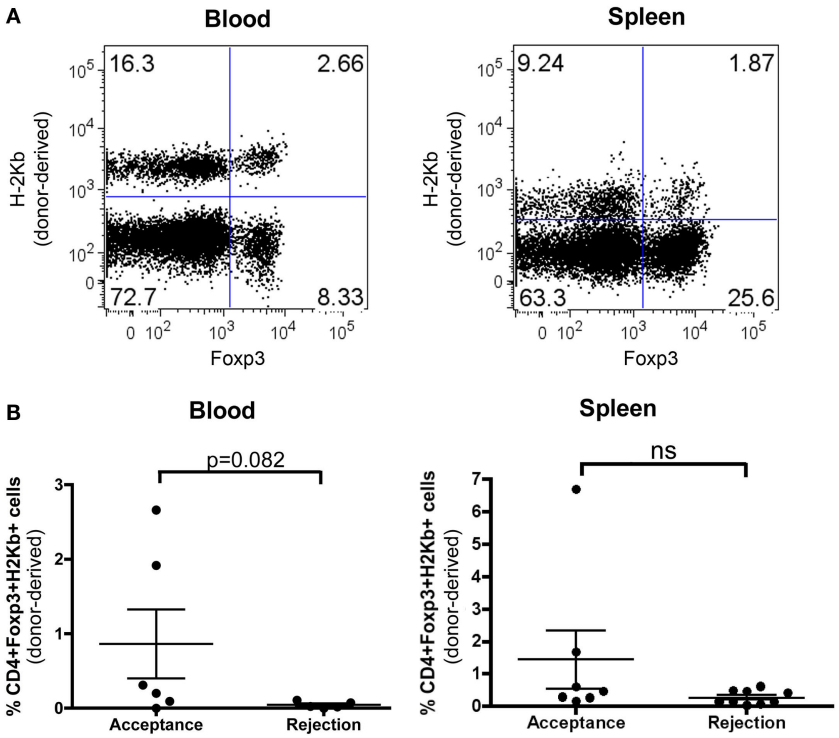

FIGURE 4 | *De novo* generation of donor Tregs in NM-BMT mice receiving RA-iTregs. **(A)** One hundred days after skin transplant, donor-derived Tregs were analyzed in peripheral blood and spleen in NM-BMT mice treated with RA-iTregs. **(B)** Percentage of CD4 + Foxp3 + H-2K^{b+} in peripheral blood and spleen in mice that accept (n = 6) or reject (n = 7) the allogeneic skin transplant. Bars represent the SE. The Mann–Whitney–Wilcoxon statistical test was used. The results represent two independent experiments.

preconditioning setting promotes mixed chimerism and significantly extends the survival of allogeneic skin graft.

CD4 + CD25 + Foxp3+ Tregs have been used previously to generate mixed chimerism and alloimmune tolerance in two different reports (25, 43). Joffre's group induced mixed chimerism by using donor-specific Tregs obtained through the expansion of splenic CD4 + CD25 + Foxp3+ cells with allogeneic dendritic cells and IL-2 in combination with 5 Gy TBI (43). Despite the positive results they obtained, the use of high-dose irradiation is critical and limits future application in humans (48, 49). In the second report, the authors tested different types of polyclonal Tregs: nTregs, iTregs, and FoxP3 transduced Tregs, in their ability to induce mixed chimerism (25). These authors induced mixed chimerism by using these Tregs in combination with Rapamycin, co-stimulation blockade with Abatacept, and anti-CD154 as non-myeloablative preconditioning, thereby avoiding the use of radiation. This work demonstrates that natural or induced Tregs allow the generation of mixed chimerism and transplantation tolerance without cytoreductive conditioning (25). Although these results are promising, translation into clinical use in humans faces two problems; first, the use of anti-CD154 is restricted to experimental procedures only, and second, the use of polyclonal Tregs may inhibit immune responses against infections or may cause higher rates of cancer. Here, we sought to improve these preconditioning protocols so that it could be translated into clinical application in humans. The protocol we developed is donor specific and avoids the use of anti-CD154, thus, diminishing the risks associated with its use and allowing its future translation to human trials (27).

The goal of transplant immunology is to provide specific-donor immunosuppression, and this specificity could be achieved by alloantigen-specific Tregs. Here, we show that RA-iTregs express immunosuppressive molecules and specifically inhibit the proliferation of effector T cells that recognized the same alloantigen. The latter observation is in agreement with previous work from our group (42, 44), where we demonstrated that RA-iTreg cells showed alloantigen-specific immunosuppressive capacity in a skin allograft model in immunodeficient mice. In support of this, work by Joffre's group show selective grafting of allogeneic bone marrow from the specificity of Tregs, whereas bone marrow from a third strain was rejected (43, 48). Moreover, in a previous report, we have shown that RA-iTreg cells are stable in time when adoptively transferred and present an unbiased homing capability (42). Thus, alloantigen-specific Tregs may establish specific tolerance while maintaining immune responsiveness to foreign antigens.

A recent article published by Wekerle's group reports that rapamycin and CTLA4Ig synergize to induce stable mixed chimerism (50). Their strategy to induce mixed chimerism is very similar to ours except that they give mice an extra dose of CTLA4Ig and they use 2 Gy of TBI. Importantly, they did not use

Tregs to induce chimerism. In our hands, we obtain stable mixed chimerism only when we add RA-iTregs. The differences between Wekerle's and our results may be due, in part, to the combination of donor-recipient mouse strains used in both studies. Although we achieved some chimerism without Tregs, it was only transient since it was lost by 7 weeks. On the other hand, Wekerle and collaborators induce donor-specific tolerance to heart allografts but they do not succeed with skin allograft as we did, which is considered to be more stringent than the heart transplant. Here, we developed a powerful long-term operational tolerance protocol to donor-specific skin allograft, surpassing minor and major antigens barriers to produce mixed chimerism induction.

The protocol we used in this work to induce mixed chimerism allows the reconstitution of all hematopoietic cell populations frequently found in peripheral blood from the donor. The percentage of lymphocytes remains stable or increases over time while macrophages and granulocytes tend to decrease. The reasons why myeloid cells change over time have not been elucidated; however, the establishment of tolerance has been associated with the presence of donor T cells (51). It has been shown that tolerance induced by mixed chimerism can be reversed by depleting CD4+ T cells (52, 53). Thus, the decrease in the myeloid populations could be an irrelevant factor in the establishment of tolerance although this should not be entirely disregarded. Although we could not achieve a 100% graft survival, here, we demonstrate that mixed chimerism induced by RA-iTregs extends the life of the allogeneic skin transplant. As transplant tolerance correlates with the level of chimerism (54, 55), we could speculate that the percentage of graft survival could be increased if the transplant is performed at earlier times following the BMT.

Mice that received our conditioning protocol of NM-BMT together with RA-iTregs exhibited donor-derived Tregs. These cells were detected in blood and spleen 120 days after the transplant. Previous reports where mixed chimerism was obtained through non-myeloablative preconditioning and depletion of donor CD25+ cells in the transplanted BM, showed no transplant acceptance (56). Moreover, the depletion of donor or recipient Foxp3+ cells following skin and heart transplant induces the loss of allogeneic tolerance mediated by mixed chimerism (57). Although we observed a positive correlation between the presence of donor-derived Tregs and the acceptance of skin transplant, this correlation was not statistically significant. A complete evaluation of this donor Tregs in other tissues of mice that accepted or rejected their allografts are needed in order to determine the origin and contribution of these cells in transplant tolerance.

The translation of this protocol to induce donor-specific tolerance in humans is possible since several protocols to differentiate human CD4+ T cells into Tregs have been described (58). Therefore, the establishment of mixed chimerism through the use of donor-specific Tregs rather than general or non-specific immunosuppression could constitute a reasonable alternative in organ transplantation.

AUTHOR CONTRIBUTIONS

PR wrote the paper and performed the research. PM performed the research. YH performed the research. DS wrote the paper. MR wrote the paper. MB designed the research and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00596

REFERENCES

1. Gass AL, Emaminia A, Lanier G, Aggarwal C, Brown KA, Raffa M, et al. Cardiac transplantation in the new era. *Cardiol Rev* (2015) **23**(4):182–8. doi:10.1097/CRD.0000000000000066
2. Kloc M, Kubiak JZ, Li XC, Ghobrial RM. Pericytes, microvasular dysfunction, and chronic rejection. *Transplantation* (2015) **99**(4):658–67. doi:10.1097/TP.0000000000000648
3. Stegall MD, Gaston RS, Cosio FG, Matas A. Through a glass darkly: seeking clarity in preventing late kidney transplant failure. *J Am Soc Nephrol* (2015) **26**(1):20–9. doi:10.1681/ASN.2014040378
4. Wekerle T, Sykes M. Mixed chimerism and transplantation tolerance. *Annu Rev Med* (2001) **52**:353–70. doi:10.1146/annurev.med.52.1.353
5. Pilat N, Wekerle T. Transplantation tolerance through mixed chimerism. *Nat Rev Nephrol* (2010) **6**(10):594–605. doi:10.1038/nrneph.2010.110
6. Blaha P, Bigenzahn S, Koporc Z, Sykes M, Muehlbacher F, Wekerle T. Short-term immunosuppression facilitates induction of mixed chimerism and tolerance after bone marrow transplantation without cytoreductive conditioning. *Transplantation* (2005) **80**(2):237–43. doi:10.1097/01.TP.0000164510.25625.70
7. Kawai T, Sogawa H, Boskovic S, Abrahamian G, Smith RN, Wee SL, et al. CD154 blockade for induction of mixed chimerism and prolonged renal allograft survival in nonhuman primates. *Am J Transplant* (2004) **4**(9):1391–8. doi:10.1111/j.1600-6143.2004.00523.x
8. Kim N, Lee H, Shin J, Nam YS, Im KI, Lim JY, et al. Immune reconstitution kinetics following intentionally induced mixed chimerism by nonmyeloablative transplantation. *PLoS One* (2015) **10**(5):e0126318. doi:10.1371/journal.pone.0126318
9. Baśkiewicz-Hałasa M, Hałasa M, Grzegrzółka R, Piecyk K, Rogińska D, Lejkowska R, et al. Mixed chimerism induction influences cytokine release from chimeric mice cells. *Transpl Immunol* (2013) **29**(1–4):130–7. doi:10.1016/j.trim.2013.09.003
10. Domenig C, Sanchez-Fueyo A, Kurtz J, Alexopoulos SP, Mariat C, Sykes M, et al. Roles of deletion and regulation in creating mixed chimerism and allograft tolerance using a nonlymphoablative irradiation-free protocol. *J Immunol* (2005) **175**(1):51–60. doi:10.4049/jimmunol.175.1.51
11. Yamada Y, Ochiai T, Boskovic S, Nadazdin O, Oura T, Schoenfeld D, et al. Use of CTLA4Ig for induction of mixed chimerism and renal allograft tolerance in nonhuman primates. *Am J Transplant* (2014) **14**(12):2704–12. doi:10.1111/ajt.12936
12. Sayegh MH, Fine NA, Smith JL, Rennke HG, Milford EL, Tilney NL. Immunologic tolerance to renal allografts after bone marrow transplants from the same donors. *Ann Intern Med* (1991) **114**(11):954–5. doi:10.7326/0003-4819-114-11-954

13. Helg C, Chapuis B, Bolle JF, Morel P, Salomon D, Roux E, et al. Renal transplantation without immunosuppression in a host with tolerance induced by allogeneic bone marrow transplantation. *Transplantation* (1994) **58**(12):1420–2.
14. Scandling JD, Busque S, Dejbakhsh-Jones S, Benike C, Sarwal M, Millan MT, et al. Tolerance and withdrawal of immunosuppressive drugs in patients given kidney and hematopoietic cell transplants. *Am J Transplant* (2012) **12**(5):1133–45. doi:10.1111/j.1600-6143.2012.03992.x
15. Scandling JD, Busque S, Dejbakhsh-Jones S, Benike C, Millan MT, Shizuru JA, et al. Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med* (2008) **358**(4):362–8. doi:10.1056/NEJMoa074191
16. Hale DA, Gottschalk R, Umemura A, Maki T, Monaco AP. Establishment of stable multilineage hematopoietic chimerism and donor-specific tolerance without irradiation. *Transplantation* (2000) **69**(7):1242–51. doi:10.1097/00007890-200004150-00008
17. Umemura A, Monaco AP, Maki T. Expression of MHC class II antigen is essential in tolerance induction by donor bone marrow cell in antilymphocyte serum-treated and rapamycin-treated mice. *Transplant Proc* (2001) **33**(1–2):148. doi:10.1016/S0041-1345(00)01947-3
18. Ildstad ST, Sachs DH. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature* (1984) **307**(5947):168–70. doi:10.1038/307168a0
19. Colson YL, Li H, Boggs SS, Patrene KD, Johnson PC, Ildstad ST. Durable mixed allogeneic chimerism and tolerance by a nonlethal radiation-based cytoreductive approach. *J Immunol* (1996) **157**(7):2820–9.
20. Pan H, Wang L, Zhang X, Zhang G, Mai H, Han Y, et al. Rapamycin, mycophenolate mofetil, methylprednisolone, and cytotoxic T-lymphocyte-associated antigen 4 immunoglobulin-based conditioning regimen to induce partial tolerance to hind limb allografts without cytoreductive conditioning. *Transplant Proc* (2008) **40**(5):1714–21. doi:10.1016/j.transproceed.2008.03.152
21. Sykes M, Szot GL, Swenson KA, Pearson DA. Induction of high levels of allogeneic hematopoietic reconstitution and donor-specific tolerance without myelosuppressive conditioning. *Nat Med* (1997) **3**(7):783–7. doi:10.1038/nm0797-783
22. Tomita Y, Sachs DH, Khan A, Sykes M. Additional monoclonal antibody (mAB) injections can replace thymic irradiation to allow induction of mixed chimerism and tolerance in mice receiving bone marrow transplantation after conditioning with anti-T cell mABs and 3-Gy whole body irradiation. *Transplantation* (1996) **61**(3):469–77. doi:10.1097/00007890-199602150-00028
23. Nomoto K, Yung-Yun K, Omoto K, Umesue M, Murakami Y, Matsuzaki G, et al. Tolerance induction in a fully allogeneic combination using anti-T cell receptor-alpha beta monoclonal antibody, low dose irradiation, and donor bone marrow transfusion. *Transplantation* (1995) **59**(3):395–401. doi:10.1097/00007890-199502150-00015
24. Wekerle T, Kurtz J, Ito H, Ronquillo JV, Dong V, Zhao G, et al. Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. *Nat Med* (2000) **6**(4):464–9. doi:10.1038/74731
25. Pilat N, Baranyi U, Klaus C, Jaeckel E, Mpofu N, Wrba F, et al. Treg-therapy allows mixed chimerism and transplantation tolerance without cytoreductive conditioning. *Am J Transplant* (2010) **10**(4):751–62. doi:10.1111/j.1600-6143.2010.03018.x
26. Pilat N, Sayegh MH, Wekerle T. Costimulatory pathways in transplantation. *Semin Immunol* (2011) **23**(4):293–303. doi:10.1016/j.smim.2011.04.002
27. Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med* (2000) **6**(2):114. doi:10.1038/72162
28. Li XL, Ménoret S, Le Mauff B, Angin M, Anegon I. Promises and obstacles for the blockade of CD40-CD40L interactions in allotransplantation. *Transplantation* (2008) **86**(1):10–5. doi:10.1097/TP.0b013e31817c4b97
29. Goverman J, Woods A, Larson L, Weiner LP, Hood L, Zaller DM. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell* (1993) **72**(4):551–60. doi:10.1016/0092-8674(93)90074-Z
30. Bianchi T, Pincus LB, Wurbel MA, Rich BE, Kupper TS, Fuhlbrigge RC, et al. Maintenance of peripheral tolerance through controlled tissue homing of antigen-specific T cells in K14-mOVA mice. *J Immunol* (2009) **182**(8):4665–74. doi:10.4049/jimmunol.0803628
31. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol* (2003) **3**(3):199–210. doi:10.1038/nri1027
32. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* (1998) **188**(2):287–96. doi:10.1084/jem.188.2.287
33. Piccirillo CA, Shevach EM. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. *J Immunol* (2001) **167**(3):1137–40. doi:10.4049/jimmunol.167.3.1137
34. Golshayan D, Jiang S, Tsang J, Garin MI, Mottet C, Lechler RI. In vitro-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance. *Blood* (2007) **109**(2):827–35. doi:10.1182/blood-2006-05-025460
35. Sela U, Olds P, Park A, Schlesinger SJ, Steinman RM. Dendritic cells induce antigen-specific regulatory T cells that prevent graft versus host disease and persist in mice. *J Exp Med* (2011) **208**(12):2489–96. doi:10.1084/jem.20110466
36. Zhang X, Li M, Lian D, Zheng X, Zhang ZX, Ichim TE, et al. Generation of therapeutic dendritic cells and regulatory T cells for preventing allogeneic cardiac graft rejection. *Clin Immunol* (2008) **127**(3):313–21. doi:10.1016/j.clim.2008.01.013
37. Kingsley CI, Karim M, Bushell AR, Wood KJ. CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J Immunol* (2002) **168**(3):1080–6. doi:10.4049/jimmunol.168.3.1080
38. Teng L, Liu L, Su Y, Yuan X, Li J, Fu Q, et al. Suppression of alloimmunity in mice by regulatory T cells converted with conditioned media. *J Surg Res* (2011) **171**(2):797–806. doi:10.1016/j.jss.2010.03.044
39. Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity* (2009) **30**(5):656–65. doi:10.1016/j.immuni.2009.04.006
40. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* (2002) **99**(10):3493–9. doi:10.1182/blood.V99.10.3493
41. Xia G, Shah M, Luo X. Prevention of allograft rejection by amplification of Foxp3(+)CD4(+)CD25(+) regulatory T cells. *Transl Res* (2009) **153**(2):60–70. doi:10.1016/j.trsl.2008.12.001
42. Moore C, Tejon G, Fuentes C, Hidalgo Y, Bono MR, Maldonado P, et al. Alloreactive regulatory T cells generated with retinoic acid prevent skin allograft rejection. *Eur J Immunol* (2015) **45**(2):452–63. doi:10.1002/eji.201444743
43. Joffre O, Santolaria T, Calise D, Al Saati T, Hudrisier D, Romagnoli P, et al. Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. *Nat Med* (2008) **14**(1):88–92. doi:10.1038/nm1688
44. Yu P, Xiong S, He Q, Chu Y, Lu C, Ramlogan CA, et al. Induction of allogeneic mixed chimerism by immature dendritic cells and bone marrow transplantation leads to prolonged tolerance to major histocompatibility complex disparate allografts. *Immunology* (2009) **127**(4):500–11. doi:10.1111/j.1365-2567.2009.03057.x
45. Eto M, Hackstein H, Kaneko K, Nomoto K, Thomson AW. Promotion of skin graft tolerance across MHC barriers by mobilization of dendritic cells in donor hemopoietic cell infusions. *J Immunol* (2002) **169**(5):2390–6. doi:10.4049/jimmunol.169.5.2390
46. Abadja F, Sarraj B, Ansari MJ. Significance of T helper 17 immunity in transplantation. *Curr Opin Organ Transplant* (2012) **17**(1):8–14. doi:10.1097/MOT.0b013e32834ef4e4
47. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. *Transplantation* (2012) **93**(1):1–10. doi:10.1097/TP.0b013e31823cab44
48. Pilat N, Hock K, Wekerle T. Mixed chimerism through donor bone marrow transplantation: a tolerogenic cell therapy for application in organ transplantation. *Curr Opin Organ Transplant* (2012) **17**(1):63–70. doi:10.1097/MOT.0b013e32834ee68b
49. Vriesendorp HM. Aims of conditioning. *Exp Hematol* (2003) **31**(10):844–54. doi:10.1016/S0301-472X(03)00229-7
50. Pilat N, Klaus C, Schwarz C, Hock K, Oberhuber R, Schwaiger E, et al. Rapamycin and CTLA4Ig synergize to induce stable mixed chimerism without the need for CD40 blockade. *Am J Transplant* (2015) **15**(6):1568–79. doi:10.1111/ajt.13154

51. Umemura A, Morita H, Li XC, Tahan S, Monaco AP, Maki T. Dissociation of hemopoietic chimerism and allograft tolerance after allogeneic bone marrow transplantation. *J Immunol* (2001) **167**(6):3043–8. doi:10.4049/jimmunol.167.6.3043
52. Taylor PA, Lees CJ, Waldmann H, Noelle RJ, Blazar BR. Requirements for the promotion of allogeneic engraftment by anti-CD154 (anti-CD40L) monoclonal antibody under nonmyeloablative conditions. *Blood* (2001) **98**(2):467–74. doi:10.1182/blood.V98.2.467
53. Adams AB, Durham MM, Kean L, Shirasugi N, Ha J, Williams MA, et al. Costimulation blockade, busulfan, and bone marrow promote titratable macrochimerism, induce transplantation tolerance, and correct genetic hemoglobinopathies with minimal myelosuppression. *J Immunol* (2001) **167**(2):1103–11. doi:10.4049/jimmunol.167.2.1103
54. Mucida D, Pino-Lagos K, Kim G, Nowak E, Benson MJ, Kronenberg M, et al. Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naive T cells. *Immunity* (2009) **30**(4):471–2. doi:10.1016/j.immuni.2009.03.008
55. Lin JY, Tsai FC, Wallace CG, Huang WC, Wei FC, Liao SK. Optimizing chimerism level through bone marrow transplantation and irradiation to induce long-term tolerance to composite tissue allotransplantation. *J Surg Res* (2012) **178**(1):487–93. doi:10.1016/j.jss.2012.02.064
56. Bigenzahn S, Blaha P, Koporc Z, Pree I, Selzer E, Bergmeister H, et al. The role of non-deletional tolerance mechanisms in a murine model of mixed chimerism with costimulation blockade. *Am J Transplant* (2005) **5**(6):1237–47. doi:10.1111/j.1600-6143.2005.00862.x
57. Shinoda K, Akiyoshi T, Chase CM, Farkash EA, Ndishabandi DK, Raczek CM, et al. Depletion of foxp3(+) T cells abrogates tolerance of skin and heart allografts in murine mixed chimeras without the loss of mixed chimerism. *Am J Transplant* (2014) **14**(10):2263–74. doi:10.1111/ajt.12851
58. Imai A, Sugita S, Kawazoe Y, Horie S, Yamada Y, Keino H, et al. Immunosuppressive properties of regulatory T cells generated by incubation of peripheral blood mononuclear cells with supernatants of human RPE cells. *Invest Ophthalmol Vis Sci* (2012) **53**(11):7299–309. doi:10.1167/iovs.12-10182

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REVIEW
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Regulatory T cells: serious contenders in the promise for immunological tolerance in transplantation

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Regulatory T cells (Tregs) play an important role in immunoregulation and have been shown in animal models to promote transplantation tolerance and curb autoimmunity following their adoptive transfer. The safety and potential therapeutic efficacy of these cells has already been reported in Phase I trials of bone-marrow transplantation and type I diabetes, the success of which has motivated the broadened application of these cells in solid-organ transplantation. Despite major advances in the clinical translation of these cells, there are still key questions to be addressed to ensure that Tregs attest their reputation as ideal candidates for tolerance induction. In this review, we will discuss the unique traits of Tregs that have attracted such fame in the arena of tolerance induction. We will outline the protocols used for their *ex vivo* expansion and discuss the future directions of Treg cell therapy. In this regard, we will review the concept of Treg heterogeneity, the desire to isolate and expand a functionally superior Treg population and report on the effect of differing culture conditions. The relevance of Treg migratory capacity will also be discussed together with methods of *in vivo* visualization of the infused cells. Moreover, we will highlight key advances in the identification and expansion of antigen-specific Tregs and discuss their significance for cell therapy application. We will also summarize the clinical parameters that are of importance, alongside cell manufacture, from the choice of immunosuppression regimens to the number of injections in order to direct the success of future efficacy trials of Treg cell therapy. Years of research in the field of tolerance have seen an accumulation of knowledge and expertise in the field of Treg biology. This perpetual progression has been the driving force behind the many successes to date and has put us now within touching distance of our ultimate success, immunological tolerance.

Keywords: tregs, tolerance, transplantation, cellular therapy, clinical trials

Introduction

Improvements in surgical techniques and the institution of T-cell directed immunosuppressive agents in the clinical transplantation of solid organs have seen remarkable advances now forming part of a well-established treatment for end-stage failure of several major organs. However, despite vast improvements in short-term survival rates, long-term graft survival remains poor

owing to episodes of chronic rejection and the relative toxicity associated with life-long immunosuppression (1). The constant proportion of transplanted organs lost each year, necessitating re-transplantation, in a climate of donor organ shortage, places further strain on an already saturated transplant waiting list. With this in mind, the current standing of immunosuppression in transplantation is far from ideal. As such, the ultimate goal following transplantation is to induce immunological tolerance by "re-educating" the host's immune response, permitting allograft acceptance without the need for pharmacological immunosuppression, thus ensuring long-term graft survival and abolishing drug-toxicity simultaneously.

The proposal of a distinct subset of T cells able to suppress immune responses was first put forward in the 1970s, which led to scientists around the world scouring for the existence of these "suppressor" T cells (2). It was not till the mid 90s when a thymic-derived lymphocytic population, coined regulatory T cells (Tregs), were defined. Subsequent years saw accumulating evidence certifying the therapeutic potential of these cells in preventing alloimmunity, explored in animal models, presenting Tregs as ideal candidates for use in tolerance-promoting protocols. Such an evolution in the field has since provided the impetus for the development of robust Treg manufacturing plans for the isolation and expansion of a functional and stable Treg product.

Here, we dissect the characterization and operation of these cells and outline strategies employed for their isolation and *ex vivo* expansion, which in turn have inspired their therapeutic application in bone-marrow transplantation (BMT), type-1 diabetes and, more recently, solid-organ transplantation.

Regulatory T cells

Tregs constitute approximately 1–3% of circulating $CD4^+$ T cells in the periphery (3) and have been characterized by the high and stable expression of surface interleukin-2 receptor α chain (IL-2Rα, $CD25^{hi}$) (4).

Initially, Tregs were conventionally characterized in accordance with their site of differentiation, namely thymus-derived natural Tregs (tTregs) and peripherally induced Tregs (pTregs), alongside their *in vitro* counterparts, commonly referred to as iTregs (5) **(Figure 1)**. tTregs, from here on referred to as Tregs, are spawned from negatively selected thymocytes, whereas the conditions favoring the generation of pTregs include suboptimal dendritic cell (DC) activation, sub-immunogenic doses of agonist peptide, mucosal administration of peptide, and antigenic encounter in a pro-tolerogenic environment, such as in the presence of interleukin-10 (IL-10), transforming growth factor-β (TGF-β), interleukin-2 (IL-2), and retinoic acid (6).

There are at least two well-defined populations of pTregs; Th3, first identified from their role in oral tolerance through the secretion of TGF-β (7), and Tr1, characterized on the basis of their role in preventing autoimmune colitis (8) and their ability to secrete large amounts of IL-10 (9, 10). As such, pTregs are implicated in the induction of oral and gut tolerance (11) and generated in chronically inflamed and transplanted tissues (12).

Of note, the phenotypic distinction of thymic and peripherally derived Tregs has not been clearly established, posing challenges in classifying the definitive proportions of these two subsets in secondary lymphoid organs and non-lymphoid tissues alike. In mice, neuropilin (Nrp-1) expressed on tTregs can differentiate these cells from their peripherally derived counterparts, which do not express this molecule (13, 14). However, this distinction does not hold true for human Tregs.

While Tregs have been crudely accrued into these populations, even within these factions, Tregs still exist in a highly organized, heterogeneous state. Various different surface and intracellular immunological markers have been studied, defining Tregs based on their functional characteristics, migration, and lineage plasticity.

In line with this, further characterization and understanding of Treg cell biology came from the discovery of FOXP3, an intracellular transcription factor known to play a crucial role in the development and function of Tregs in a highly specific manner (15). Rare mutations of the *FOXP3* gene have been linked with the development of immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), leading to organ-specific autoimmune diseases including insulin-dependent diabetes mellitus and various hematological disorders (15). Furthermore, the importance of FOXP3 in the safeguarding of Treg phenotype and function has been reiterated in studies where a loss/diminution of FOXP3 expression in Tregs has been shown to affect the competency of these cells acquiring certain effector T cell properties, including production of cytokines, such as IL-2, IL-4, IL-17, and IFN-γ (16).

Additionally, while *FOXP3* has been termed a "master control gene," specifically with regards to Treg development, its expression is not uniformly homogenous. In contrast to mice, where *foxp3* is expressed exclusively on Tregs, in humans, increasing evidence has shown that effector cells can transiently express FOXP3, with no associated regulatory activity. Based on such studies and taking also into account its intracellular expression, this marker in isolation cannot be considered to be entirely sufficient in demarcating human Tregs (17).

However, reports have commented on the inverse correlation between the expression of the α-chain of the IL-7 receptor, CD127, and FOXP3 expression with respect to Treg functional suppressive capabilities (18). As such, the combination of CD25, FOXP3, and CD127 are considered to be the most stringent markers in defining Tregs in the research setting.

Additionally, following the recent discovery of naïve suppressive $FOXP3^+$ cells ($CD45RA^+$) present in the cord blood and in adult blood, and $FOXP3^+$ cells, which express a memory-like phenotype ($CD45RA^-$), it has been proposed that three phenotypically and functionally distinct sub-populations based on the differential expression of CD25, FOXP3, and CD45RA can be defined: population I ($CD25^{++}FOXP3^+CD45RA^+$) classified as resting Tregs, population II ($CD25^{+++}FOXP3^{hi}CD45RA^-$) termed activated Tregs, and population III ($CD25^{++}FOXP3^+CD45RA^-$), which was proposed to consist of non-suppressive $FOXP3^{lo}$ cells (19). Further analysis of the three populations by Miyara et al. revealed that population I and II were both able to suppress *in vitro* with population II displaying a higher expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), a mechanism proposed for Treg suppressor function (**Figure 2**),

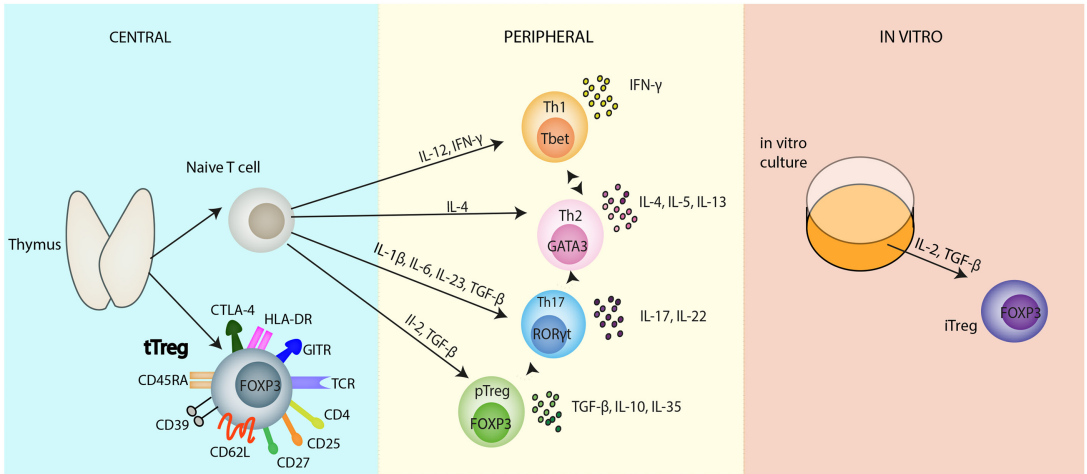

FIGURE 1 | Regulatory T cell populations. Selection of naïve CD4+ T cells and natural Tregs occurs in the thymus. Thymic-derived natural Tregs (tTregs), the main focus of this review, have been reported to express a variety of activation and functional markers as depicted in the diagram. Naïve CD4+ T cells, subsequently, can differentiate into several different T cell subsets: Th1, Th2, Th17, induced Tregs, in the periphery, all heralding distinct immunological roles. These differentiation programs are controlled by different cytokines and each separate CD4+ T cell subset can be identified from their lineage-specific transcription factors responsible for the regulation and maintenance of their individual functions; T-bet (Th1 cells), GATA3 (Th2 cells), RORγt (Th17 cells), FOXP3 (Tregs). Each subset has its own immunological role *in vivo*: Th1 cells secrete IFNγ, controlling immunity to foreign pathogens. Th2 cells produce various cytokines including: IL-4, IL-5, IL-13, IL-10, which are primarily involved in promoting humoral immunity, protecting against infection. Th17 cells produce predominantly the inflammatory cytokine, IL-17, and play an important role in controlling pathogens especially at environmental surfaces and the cytokine, IL-22. Despite the apparent terminal differentiation of all these cells, they cannot be considered to be committed to one cell fate. Lineage plasticity following differentiation is depicted by the dotted arrows between the cells. This diagram is far from comprehensive; it is most likely that the future will see various changes and additions to this diagram concerning the differentiation of CD4+ T cells. *In vitro* generation of Tregs in the presence of IL-2 and TGF-β polarizing conditions leads to the development of iTregs. Abbreviations: APC, antigen presenting cells; CD, cluster of differentiation; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; FOXP3, forkhead Box P3; IFN, interferon; IL, interleukin; IRF, interferon regulatory factor; iTreg, induced Treg; nTreg, natural Treg; pTreg, peripheral Treg; RORγt, retinoid related orphan receptor γ; T-bet, T box transcription factor; TCR, T cell receptor; TGF-β, transforming growth factor-β; Th, T helper cell; Treg, regulatory T cell.

yet were more prone to apoptosis following exertion of their suppressive function. Population III, however, was shown to be non-suppressive *in vitro* (19).

Demarcation of these three populations of Tregs was also able to depict the differentiation dynamics of FOXP3+ Tregs *in vivo*. Resting Tregs were found to upregulate their FOXP3 expression, following stimulation, and mature to terminally differentiated activated Tregs thus replenishing the apoptotic pool of activated Tregs. Miyara et al. suggested that population III had the greatest potential to differentiate into inflammatory Th17 cells, inferred from their relative IL-17 production following cytokine stimulation. The three comparative populations are found in different proportions in certain biological environments and their analysis can prove to be instrumental in identifying the immunological pathophysiology of disease and the optimal Treg subpopulation for cell therapeutic application.

It should, however, also be noted that the definitive functional characteristics of population III are controversial. Booth et al. and data from our laboratory indicated that both CD45RO+ and CD45RA+ Treg subsets are equally suppressive, population III representing a *bona fide* Treg subpopulation, bearing T cell memory markers (20). Moreover, we and others, have also reported the expression of CD161, a member of the killer cell lectin-like receptor subfamily B, on a subpopulation of human Tregs in population III, that produce IL-17 upon *in vitro* activation in the presence of IL-1β, but not IL-6. In addition, evidence has also supported the suppressive capacity of these cells (21, 22).

Above, we have outlined some of the key Treg markers of which are pertinent when considering the isolation of these cells for clinical application. However, one must be wary that the array of markers outlined in this review is far from exhaustive. For a comprehensive review of Treg markers, the reader is directed to Schmetterer et al. (23) and Povoleri et al. (6).

The mechanisms of Treg suppression still remain elusive. *In vitro* studies have demonstrated that the immunosuppressive qualities native to Tregs manifest through a variety of mechanisms, namely, modulation of APC maturation and function (24–26), anti-inflammatory cytokine production (27–30), induction of apoptosis in target cells (31, 32), and disruption of metabolic pathways (33, 34) (**Figure 2**).

Regulatory T Cells in Transplantation; Lessons Learnt From Pre-Clinical Data

The current paradigm hypothesizes that immune tolerance in transplantation is determined by a balance of Tregs over T effector cells. With this phenomenon in mind, the therapeutic potential of inducing and expanding Tregs directly *in vivo* or infusing autologous *ex vivo*-expanded Tregs represents a promising approach in the induction and maintenance of transplantation tolerance.

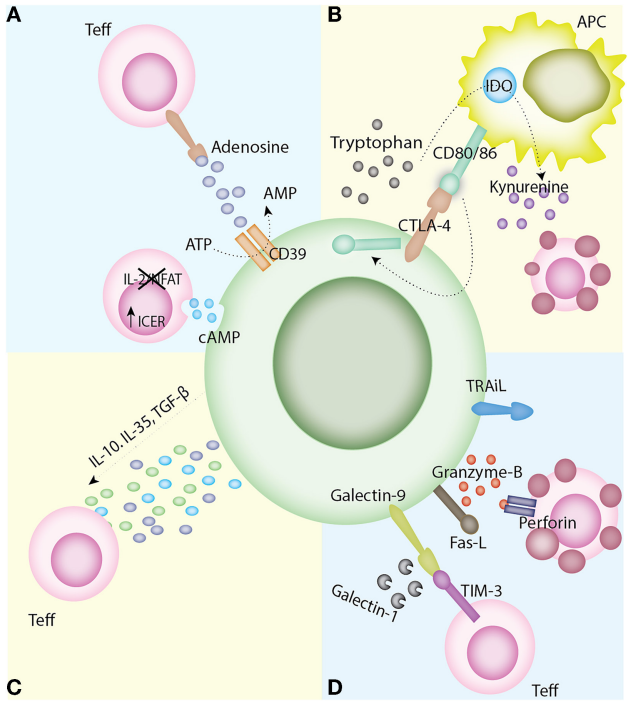

FIGURE 2 | Mechanisms of Treg suppression. **(A)** Disruption of metabolic pathways. The ectoenzymes CD39 and CD73, expressed on Tregs, result in the metabolism of ATP to AMP and in turn producing the immunoregulatory purine, adenosine. Tregs have also been found to express high levels of intracellular cAMP. This is transferred to T effector cells through gap junctions, which leads to the upregulation of ICER and in turn the inhibition of NFAT and Il-2 transcription leading to apoptosis by IL-2 deprivation. **(B)** Modulation of APC maturation and function. The interaction of CTLA-4 on Tregs with its ligand CD80/86 on APCs delivers a negative signal for T cell activation. CTLA-4's mechanism of action is varied including the capture of its APC-expressed ligands and subsequent trans-endocytosis and also the upregulation of IDO and the generation of kynurenines. **(C)** Anti-inflammatory cytokine production. The secretion of anti-inflammatory cytokines, such as IL-10, IL-35, and TGF-β, has been linked with inhibition of T cell activation *in vivo*. **(D)** Induction of apoptosis. Tregs have the capacity to directly induce apoptosis via granzyme A/B and perforin, TRAIL, the Fas/Fas-ligand pathway, the galectin-9/TIM-3 pathway, or the production of galectin-1. Abbreviations: APC, antigen presenting cell; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CD, cluster differentiation; CTLA-4-cytotoxic T lymphocyte antigen-4; DC, dendritic cells; ICER, inducible cAMP early repressor, IDO, indoleamine 2,3-dioxygenase IL, interleukin; NFAT, nuclear factor of activated T cells; TGF-β, transforming growth factor-β; TIM-3, T cell immunoglobulin and mucin domain-3; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Treg, regulatory T cells.

Series of pre-clinical rodent models of skin and cardiac transplantation demonstrated that Tregs present in the recipient at the time of transplantation are critical in the induction and maintenance of tolerance [reviewed in Ref. (35)]. Additionally, mouse models of BMT further supported the importance of adoptive Treg therapy, whereby the transfer of freshly isolated Tregs together with the bone-marrow allograft resulted in amelioration of graft versus host disease (GvHD) and facilitated engraftment (36, 37).

Moreover, adoptive transfer of Tregs has been shown to prevent rejection in other murine models of transplantation, such as pancreatic islets (38).

An issue for consideration in Treg cell therapy in transplantation is the relevance of Treg allospecificity with the selective advantage that the immunomodulatory function of these cells would be concentrated at the site of alloantigen and immune activation (39). An additional advantage of alloantigen-specific cellular therapy is that undesirable pan-suppression, resulting in increased risk of infections and cancers, is less likely to occur.

Although the indirect pathway has been implicated in acute graft rejection (40), its influence has been more closely associated with chronic allograft rejection (41). Indeed, much evidence suggests that for tolerance to occur, this is the pathway that needs to be regulated and it is this pathway of allorecognition that is used by Tregs for immunoregulation (29, 41–44).

In agreement, we have shown that using Tregs with indirect pathway anti-donor allospecificity for a single MHC class I result in the induction of donor-specific transplantation tolerance in a murine skin transplant model following thymectomy and selective T cell depletion (45). However, in a later study, we reported that Treg lines specific for directly and indirectly presented alloantigens are needed to induce indefinite survival of MHC-mismatched heart allografts, with Tregs with indirect allospecificity necessary to prevent chronic vasculopathy (46). Moreover, Joffre et al. have provided additional evidence that Tregs with direct allospecificity alone cannot protect against chronic rejections (47) supporting the notion that Tregs with both specificities are necessary to control allograft rejection.

Additional support for the use of alloantigen-specific Tregs in the transplant setting has been made available by the use of currently available humanized mouse models of allotransplantation (48–52). These models are based on the reconstitution of immunodeficient mice with human immune cells. Our group has recently shown the efficacy of human Tregs with direct allospecificity in preventing alloimmune dermal tissue injury using a humanized mouse model of skin transplantation in which only T cells have engrafted (53). The general consensus throughout these studies concluded that donor antigen-specific Tregs are more effective as compared to polyclonal Tregs.

In addition to the evidence supporting the importance of antigen-specific Tregs in preventing solid-organ rejection, after BMT donor-specific Tregs have been shown to preserve graft versus tumor activity, while inhibiting GvHD (54). However, further studies in the context of GvHD have reported that the transfer of Tregs enriched for alloantigen-specificity showed only moderately improved efficacy when compared to polyclonal Treg cell populations (55). As such, phase I clinical trials, using polyclonal Tregs, following hematopoietic stem cell transplantation have been conducted (56–58).

Such adoptive transfer experiments in rodents, therefore, have informed and instigated efforts to harness the immunoregulatory properties of these cells in novel tolerance promoting strategies in the prevention of rejection after organ transplantation. Thus, "tipping the balance" in favor of regulation by directly applying *ex vivo*-expanded Tregs is a promising strategy. In the next section, we will highlight the advances in the field in view of Treg

isolation and expansion, reviewing some of the challenges and progress to date as well as review the lessons learned from the clinical application of these cells.

Treg Manufacture for Clinical Application

Approaches for Treg Isolation

The effective implementation of Treg therapy in transplantation is dependent on Treg manufacturing plans with protocols that are compliant with good manufacturing practice (GMP).

The clinical Treg selection protocol to date in the UK has been used by our group in the new Clinical Research Facility (CRF) at Guy's Hospital and involves a combination of depletion of $CD8^+$ cells and positive selection of $CD25^+$ cells using the automated CliniMACS plus system (Miltenyi Biotec, Bisley, United Kingdom), which is centered around the concept of magnetic bead isolation. The major drawback with such a technique is that this process does not allow the selection of Tregs based on multiple parameters, an attractive prospect when trying to isolate a Treg population with the desired characteristics for cell therapeutic application.

Additionally, the lack of distinction of $CD25^{hi}$ cells, using this protocol, means that the isolation may include contaminating conventional activated T cells, explaining the reduced purity of this strategy for Treg isolation as compared to the use of the fluorescence-activated cell sorting (FACS) technique (59, 60).

Despite the CliniMACS system being the only currently available GMP compatible technology for the isolation of clinical grade Tregs in the UK, there is still much enthusiasm for clinical cell separation using FACS sorting. Although currently unclear as to which Treg subset provides the best therapeutic activity, GMP-compliant FACS sorting will open up the possibility of isolating Treg subsets with potent suppressive function, specificity, and those that are epigenetically stable.

Two different combinations of markers have been proposed to be promising for the isolation of a pure Treg population. The first seeks to isolate $CD4^+CD25^{hi}$ Tregs with the addition of an antibody to select for $CD45RA^+$ cells and so eliminate antigen experienced or memory T cells (61). Moreover, this so-called naïve Treg population yields Tregs with a greater suppressive capacity than total $CD25^{hi}$ cells (62) and have the greatest expansion potential (61).

Furthermore, it has been demonstrated that between the *FOXP3* promoter and the first exon lies a stretch of highly conserved, non-coding sequence that is differentially methylated in tTregs, pTregs, and T effectors (63, 64). This sequence, referred to as the Treg-specific demethylated (TSDR) region, is crucial at maintaining high FOXP3 expression in Tregs (65).

Additionally and in support of the isolation of the $CD45RA^+$ Treg subset for cell therapy application, supplementary evidence report that after 3 weeks of *in vitro* expansion, the $CD45RA^+$ expanded Tregs remained demethylated at the TSDR region, confirming their stability during expansion (60, 62).

The importance of isolating and expanding a stable Treg population becomes even more pertinent when considering Treg cell therapy in inflammatory/autoimmune conditions. Emerging data have highlighted that despite the strict government of FOXP3 expression, Tregs can downregulate FOXP3 in the presence of inflammatory cytokines. In agreement, Yang et al. have shown that exposure of Tregs to IL-6 and IL-1 *in vitro* results in the expression of IL-17 (66). *In vivo*, loss of FOXP3 has also been documented in the setting of autoimmune disease (67) fetal acute infections (68), TLR stimulation (69), and homeostatic proliferation (70).

Therefore, with evidence supporting the stability of $CD4^+CD25^+CD45RA^+$ Tregs, we have recently advocated the isolation and expansion of these cells for cell therapeutic application in the setting of inflammatory bowel disease (71).

Despite such studies in favor of $CD45RA^+$ cells, one drawback is that the number of naïve Tregs decline in the peripheral blood with age (72) and hence isolation based on this approach may prove to be impractical.

The second approach still uses the fundamental $CD4^+CD25^{hi}$ phenotype to isolate Tregs but also includes CD127 expression. The rationale placed on the foundation that in human Tregs, there is a reciprocal expression of CD127 and FOXP3 and thus CD127 provides a sortable surrogate marker for $FOXP3^+$ Tregs (18). Moreover, two elegant studies (48, 50) support the *in vivo* superiority of the $CD4^+CD25^+CD127^{lo}$ Tregs in regulating alloreactivity compared to Tregs isolated based on the expression of CD4 and CD25 alone.

Such studies merely highlight the importance of multiparameter separation of Tregs, using the FACS cell sorter. In this regard, the last few years have not only seen significant efforts made in obtaining the relevant regulatory approvals to integrate the FACS cell sorter into a clinical cell production process, but an immense progress in the technology to do so.

As such, a new era of clinical flow sorting has seen the recent introduction of a class of flow sorting devices, utilizing microfluidic chips instead of the classical flow-in-air droplet sorters. Unlike traditional sorting, there is no high-pressure, shearing forces, or dilution by sheath fluid, resulting in a cell processing that may preserve cell function and viability. Additionally, cells are processed in closed systems, thus eliminating the risk of contamination of the product during processing. Such a system, however, did not present itself without technical challenges including the initial slow sort speed. These have been overcome either by massive parallel sorting on a single microfluidic chip, such as in the Cytonome GigaSort System (Cytonome/ST, LLC, Boston, MA, USA) (73) or by the introduction of mechanical microvalves operating at high speed, such as the recently introduced MACS Quant Tyto (Miltenyi Biotec, Bergisch Gladbach, Germany).

Although not yet clinically approved, both machines are in principal designed for clinical cell separation processes and provide significant advantages allowing their integration into GMP-compliant production processes.

Polyclonal Treg Expansion: Optimization of Current Culture Conditions

One of the obstacles in the implementation of clinical protocols for adoptive Treg cell therapy is their relative paucity in the circulation. This means that for cellular therapy, it will almost certainly be necessary to expand these cells *ex vivo*, to clinically relevant numbers, prior to their administration. It has already

been demonstrated that Tregs can be readily expanded using anti-CD3/CD28-coated beads, supplemented with IL-2 (60, 74). However, under these circumstances, effector cells have the potential to proliferate vigorously, posing a major problem for MACS-purified $CD4^+CD25^+$ Tregs, as they are often contaminated with $CD25^+FOXP3^-$ cells. Thus, this not only puts under question the potential safety of the final product but also the efficacy. As such, much effort has, therefore, focused on optimization of culture conditions to ensure the expansion of Tregs to achieve the necessary numbers yet limit the potential expansion of contaminant cells.

Rapamycin

This immunosuppressant mechanism of action involves the inhibition of the mammalian target of rapamycin (mTOR), which is downstream of phosphatidylinositol 3-kinase (PI3K), a signaling molecule activated by CD28 or IL-2 receptor engagement in T cells (75) (**Figure 3**). Characteristically, IL-2 receptor engagement activates both PI3K-mTOR and Janus kinase-STAT pathways. However, biochemical analysis of IL-2 signaling in Tregs has shown that the PI3K–mTOR pathway is underactive, whereas the Janus Kinase–STAT pathway remains intact, suggesting that Tregs preferentially signal through the latter in turn conferring their resistance to mTOR inhibition (76). In agreement, genetic ablation and cellular experiments that demonstrate mTOR deficiency or the addition of rapamycin favor the growth and preserved function of Tregs (77, 78). Paralleling these *in vitro* observations, it has been shown that rapamycin can potentiate the ability of Tregs to inhibit transplant arteriosclerosis in a humanized mouse system (79). Furthermore, in transplant patients, the use of rapamycin-based immunosuppression is also associated with an increased proportion of Tregs as compared to patients on calcineurin inhibitors (CNI) (80, 81). Thus, by favoring Treg survival and expansion and by preventing the outgrowth of contaminating effector T cells (76, 82), rapamycin ensures the selection of a pure Treg population.

Further research has not only confirmed the unique preferential preservation of Tregs by rapamycin but has also reported its role in ensuring Treg stability. Treatment of $CD4^+CD25^{hi}FOXP3^+$ with rapamycin has been shown to inhibit the development of IL-17-producing cells and to maintain a stable Treg phenotype favoring the expansion of non-plastic Treg subsets, both *in vitro* and *in vivo* (83–85). The mechanism by which this occurs is thought to involve alterations in the epigenetic profile allowing

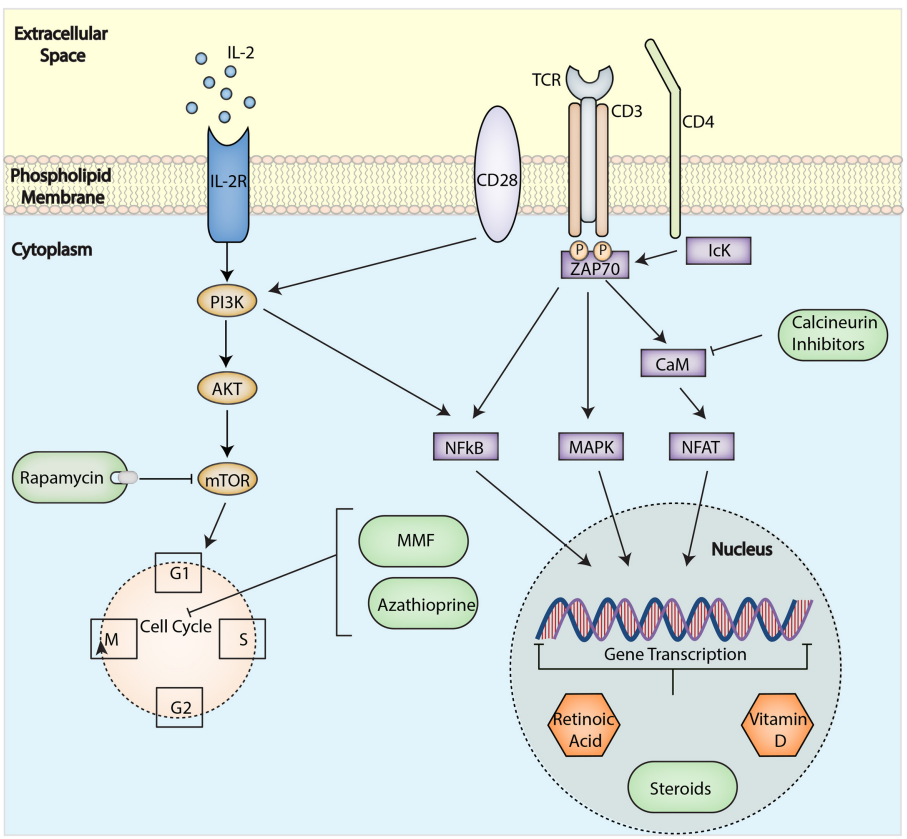

FIGURE 3 | Immunological targets for immunosuppressive drugs in T cells. Drugs can affect specific molecular pathways and control gene expression to block alloactivation of T cell after transplantation and induce tolerance. Abbreviations: TCR, T-cell receptor; mTOR, mammalian target of rapamycin; MMF, mycophenolate mofetil; PI3K, phosphatidylinositol 3-kinase; IL-2R, interleukin-2 receptor; NFkB, nuclear factor κ-light-chain-enhancer of activated B cells; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T-cells.

for the active transcription of *FOXP3* (84, 86, 87). These findings support the use of rapamycin in clinically applicable protocols for the expansion of human Tregs. We are currently testing the safety of Tregs expanded following this protocol in the CRF at Guy's Hospital in two Phase I/II clinical trials: in kidney (ONE Study: NCT02129881) and liver (ThRIL: NCT02166177) transplant patients.

Retinoic Acid and Vitamin D

Despite recent advances in Treg biology, large-scale manufacture of these cells remains challenging in view of studies reporting that even highly pure Tregs lose FOXP3 expression over time. In line with this, a study by Hoffman et al. further concluded a loss of FOXP3 expression by Tregs upon repeated anti-CD3/CD28 stimulation in culture, in turn forfeiting their stability (62). As such, recent attention has been drawn to other approaches that ensure Treg stability in culture, an example including the supplementation of cultures with all-*trans*-retinoic acid (ATRA).

Of note, natural derivatives and metabolized products of vitamin A, such as β-carotene, retinol, retinal, isotetrinoin, ATRA, and 9-*cis*-Retinoic acid all have important roles in cell differentiation, growth, and apoptosis (88). ATRA is one of the most influential molecules on T cells and has been reported to affect T cell fate by contributing to Th1/Th17 as well as Treg differentiation (89). In combination with TGF-β, ATRA has been shown to promote differentiation of naive murine and human T cells into Tregs (90–92) and, more recently, as a treatment to expand human Tregs and increase their function (84, 85, 93, 94). The molecular pathway by which ATRA favors the expansion of Tregs is not entirely clear, but it is thought to induce chromatin de-condensation recruiting histone acetyl-transferases and transcription machinery to the *FOXP3* promoter (95, 96). Furthermore, experiments in animal models have shown that deletion of ATRA nuclear receptor results in significant loss of FOXP3 expression in Tregs, suggesting that ATRA may act to stabilize FOXP3 expression (97).

However, while the use of ATRA during Treg culture heralds the expansion of a highly suppressive Treg population for cell therapy (84, 85, 94), there are concurrently major concerns regarding its use, primarily since ATRA preferentially stimulates *ex novo* generation of iTregs, which as eluded to earlier, have an unstable genetic phenotype with their plasticity influenced *in vivo* by the inflammatory conditions (87). As a consequence, the regulatory phenotype induced *in vitro* may be easily reverted into a pro-inflammatory one upon *in vivo* administration (98).

Indeed, there is a dynamic relationship between the transcription factors RORγT and FOXP3 in T cells that rules the balance between Th17 cells and Tregs. The competitive antagonism of these trans-acting factors is controlled by pathways downstream of either IL-2 (pro-Treg) or IL-1β/IL-6 signaling (pro-Th17) (98). For this reason, an intense inflammatory condition may fully override ATRA-mediated FOXP3 expression and revert a protective Treg preparation into a potentially harmful immune response (87, 98).

Another potential supplementary candidate in Treg expansion that has attracted much attention is Vitamin D, with growing evidence supporting the immunomodulatory roles of this vitamin and its importance in the induction and maintenance of FOXP3$^+$ Tregs *in vivo*. As such, studies have highlighted that serum concentrations of vitamin D positively correlate with the number and frequency of FOXP3$^+$ Treg cells in the peripheral blood of patients (99–102). However, the mechanism by which vitamin D controls the generation/expansion of Tregs is not completely clear, with vitamin D concentrations playing differing roles (102, 103). It appears that high non-physiological concentration (10^{-6} M) of Vitamin D induces IL-10 production by CD4$^+$ T cells, providing an explanation for the immune-modulating and Treg-promoting ability of these settings. Instead, at a more physiological concentration (10^{-7} M), vitamin D, together with TGF-β, favors the expansion of a highly suppressive FOXP3$^+$ Tregs (102, 103).

The past few years have seen considerable effort focused on defining an optimal technique to isolate and expand Tregs from peripheral or cord blood. The studies outlined above highlight the future research directions in view of devising protocols that incorporate the use of these reagents in the expansion of an optimal Treg population for cell therapy application.

Alloantigen-Specific Regulatory T Cell Expansion

In view of the wealth of animal data from our laboratory (49, 53, 104) and others (47, 48, 55) in support of the importance of antigen-specific Tregs in the setting of solid-organ transplantation, efforts have been directed at the generation of antigen-specific Tregs for cellular therapy in this setting.

To date, the generation and expansion of alloantigen-specific Tregs have proved to be an arduous task, in particular Tregs with indirect allospecificity. Recently, there have been advances in the propagation of Tregs with direct allospecificity using donor APCs, such as DCs and unfractionated peripheral blood mononuclear cells (PBMCs) (53, 105–107). In this regard and in a previous study, we screened human Tregs for activation markers following stimulation with allogeneic peripheral blood or dermal CD1c$^+$ DCs (53). We subsequently defined the upregulation of CD69 and CD71 by Tregs, at 3–5 days after activation, delineating the alloantigen-specific Tregs. Furthermore, we were able to comment on the suppressive superiority of these antigen-specific Tregs as compared to polyclonally expanded Tregs.

Moreover, recent reports have highlighted the effectiveness of CD40L-activated B cells in the induction and expansion of antigen-specific Tregs *in vitro* (108–110). As such and in collaboration with colleagues at University of California (UCSF), we have shown the clinical grade manufacture of Tregs against allogeneic human leukocyte antigen (HLA) of the donor (104). Purified Tregs were stimulated *ex vivo* with CD40L-activated allogeneic B cells, followed by subsequent expansion using anti-CD3/anti-CD28-coated beads with the addition of IL-2. By employing this protocol, we were able to demonstrate not only the successful expansion (300- to 500-fold) but also the alloreactivity of the expanded Tregs against the donor antigen. We further demonstrated the antigen-specific suppressive function of the expanded Tregs in protecting against alloimmune-mediated skin damage in a humanized mouse model of transplantation.

The manufacture of Tregs, using this protocol, is currently being tested as part of a clinical trial at UCSF (NCT02188719).

An alternative approach has been to engineer Tregs with the ability to target specific antigens by expressing antigen-specific TCRs (111) or taking advantage of the growing field of Chimeric Antigen Receptors (CAR) (112). To date, most of the CAR studies have focused on tumor antigen-specific cytotoxic $CD8^+$ T lymphocytes for the treatment of human cancers. However, several studies in animals suggest that CAR-expressing Tregs can be efficacious in preventing experimental autoimmune encephalitis (EAE) (113) or colitis (114). More recent work from our group has set out to investigate the efficacy of CAR-expressing Tregs in the setting of transplantation.

It is also pertinent to note that given the experimental evidence detailing the synergy of direct and indirect Tregs in the setting of transplantation tolerance, considerable efforts have been concentrated on generating and expanding Tregs with indirect allospecificity to further assist in this endeavor (115, 116). In contrast to the definitive selective expansion of Tregs with direct allospecificity from the existing repertoire, studies have demonstrated the difficulties in generating Tregs with indirect allospecificity using the same APCs used for the generation of Tregs with direct allospecificity. TCR-transduction was used by us to confer indirect allospecificity and we have demonstrated both in skin and heart transplants, the efficacy of Tregs with such a specificity. In addition, we have shown the advantage of conferring indirect alloreactivity during the expansion of direct alloreactive Tregs so as to generate Tregs with dual specificity, with promising results (111, 117).

Despite the on-going efforts to develop protocols for the manufacture and *ex vivo* expansion of allospecific Tregs, it is pertinent to note that our initial data highlighted that a highly pure population of Tregs was essential prior to their allospecific *ex vivo* expansion for this to be a success (104). As such and in view of the lack of GMP-compliant sorting technology in the UK, the application of antigen-specific Tregs in trials of Treg cell therapy in the UK have not been possible. However, with the upcoming installation of a GMP-compliant cell sorter into our facilities, efforts will be directed toward the generation of an optimal precursor population of antigen-specific Tregs for cellular therapy in the near future.

Tregs Therapy in Solid-Organ Transplantation; Our Experience to Date

The results of the trials to date have highlighted the favorable safety profile of freshly isolated and polyclonally expanded Tregs with varied reports of efficacy (56–58, 118) (**Table 1**). As a result, the prospects of Treg adoptive cell therapy are now widely recognized with the information gleaned from these preliminary trials now guiding the clinical progression of these cells into the realms of organ transplantation.

In this regard, Yamashita et al. recently reported the first trial of donor alloantigen-specific Tregs in patients undergoing living donor liver transplantation (119). Here, iTregs were generated whereby recipient PBMCs were co-cultured with irradiated donor PBMCs in the presence of costimulatory blockade. Subsequent administration of $0.6–2.6 \times 10^9$ iTregs in splenectomized patients, concurrently receiving cyclophosphamide, was found to be not only safe in this setting but also enabled the withdrawal of immunosuppression in 6 out of the 10 patients recruited.

As alluded to earlier, the last few years has also seen the start of two clinical trials of Treg immunotherapy in solid-organ transplantation at King's College London, the ONE Study (NCT02129881) and ThRIL (NCT02166177).

The ONE study is a multicenter Phase I/II study funded by the European Union FP7 program, investigating the safety of and potential efficacy of infusing *ex vivo*-expanded Tregs, among other regulatory cells. It is a dose escalation trial, designed to assess doses of 1, 3, 6, 10×10^6 Tregs/kg, injected at 5 days after transplantation so as to determine the maximum-tolerated dose. Of importance, patients, receiving the cell products at the different centers will be on a similar immunosuppression regimen that includes prednisolone, tacrolimus, and mycophenolate mofetil (MMF). This will enable the direct comparison of transplant outcomes between the varied cell products tested as part of the ONE study.

Although the primary goal of the ONE study is to assess safety, the production feasibility of each of the cell products, at the varied doses, will also be assessed. In this regard, we have successfully produced the final product that to date has enabled three patients to be dosed at 1×10^6/kg, a further three patients at 3×10^6/kg and one patient at 6×10^6/kg. Five more patients remain to be dosed, two at 6×10^6 and three at 10×10^6. The final Treg product manufactured by us has been used to treat patients recruited at Oxford University and by Guy's Hospital.

ThRIL (NCT02166177) is a combined Phase I/IIa clinical trial of Treg immunotherapy in the setting of liver transplantation where the safety, tolerability, and efficacy of 1×10^6/kg and 4.5×10^6/kg of polyclonally expanded Tregs will be assessed with thymoglobulin and an mTOR-inhibitor-based immunosuppression regimen. ThRIL is currently in the recruitment stage and to date, we have dosed the first patient for this trial.

The near future will see the reporting of these trials, which will focus primarily on the safety of the injected cells, but will also speculate on their relative therapeutic efficacy, with reference to graft survival and supplementary biochemical and immunological markers of tolerance, in a bid to support larger Phase II/III studies. The success of such trials and the outlook of Treg therapy as an entirety will be defined from effective and informative clinical trial designs with adherence to hard efficacy end points. Thus, key issues will need to be addressed prior to the design of such trials including adjunct immunosuppressive regimens, the timing and number of injections, the dose of Tregs with the desired specificity, and the trafficking properties of the infused cells.

Tregs Immunotherapy; Future Directions

Immunosuppression and Tregs

Despite the initial confidence in adoptive Treg cell therapy as a self-sufficient entity, experimental data have shown that the efficacy of Treg therapy requires a favorable *in vivo* environment,

TABLE 1 | Clinical trials of Treg immunotherapy.

Clinical trial number	Investigators	Setting	Patients recruited	Isolation	Treg doses	Study overview and results	Reference
N/A	Trzonkowski et al.	GVHD adult	2	FACS: $CD4^+CD25^+CD127^-$	1×10^5 to 3×10^6/kg	The first patient had chronic GvHD 2 years post BMT. After receiving 0.1×10^6/kg FACS purified *ex vivo* expanded Tregs from the donor, the patient was successfully withdrawn from immunosuppression without evidence of recurrence. The second patient had acute GvHD at 1-month post transplantation, treated with several infusions of expanded donor Tregs. Despite the initial and transitory improvement, the disease progressed and ultimately resulted in the patient's death	(58)
NCT00602693	Brunstein, McMillan, Blazar (2010)	GvHD adult	23	CliniMACS: $CD25^+$	0.1, 0.3, 1, and 3×10^6/kg	Tregs were isolated from a third party UCB graft and expanded polyclonally with anti-CD3/CD28 coated beads and recombinant IL-2 over a period of 18 days. Patients received expanded Tregs at doses ranging from 1×10^5/kg to 30×10^5/kg. Targeted Treg dose was only achieved in 74% of cases. Compared with the 108 historical controls, there was a reduced incidence of grades II–IV acute GvHD (from 61–43%; $p = 0.05$), although the overall incidence of GvHD was not significantly different.	(56)
N/A	Di Ianni et al.	GvHD adult	28	CliniMACS: $CD4^+CD25^+$	$2–4 \times 10^6$/kg	Patients received donor Tregs without *ex vivo* expansion and donor effector T cells (Teff) without any other adjuvant immunosuppression. Different dose regimens were used, ranging from 5×10^5/kg Teffs with 2×10^6/kg Tregs to 2×10^6/kg Teffs with 4×10^6/kg Tregs. As two patients receiving the latter regimen developed acute GvHD, compared with none of the other patients, the dose of 1×10^6/kg Teffs with 2×10^6/kg Tregs was reported to be safe. Patients receiving Tregs demonstrated accelerated immune reconstitution, reduced CMV reactivation, and a lower incidence of tumor relapse and GvHD when compared to historical controls. Disappointing patient survival was reported with only 13 out of the 26 patients surviving	(57)
N/A	Marek-Trzonkowska et al.	Type-I diabetes children	12	FACS: $CD4^+CD25^+CD127^-$	$10–20 \times 10^6$/kg	One year follow-up of 12 children with Type-I diabetes, treated with autologous-expanded *ex vivo* Tregs. Patients received either a single or double Treg infusion up to a total dose of 30×10^6/kg. The data supported the safety of the infused Tregs, with 8/12 treated patients requiring lower requirements of insulin, with two children completely insulin independent at 1 year	(118)
NCT01210664	Bluestone et al.	Type-I diabetes adult	14	FACS: $CD4^+CD25^+CD127^-$	$5 \times 10^6–2.6 \times 10^9$/kg	Infusion of 14 type-I diabetic patients with *ex vivo*-expanded Tregs (FACS purified and two rounds of anti-CD3/anti-CD28 stimulation). The first cohort of patients received 0.05×10^8 cells, the second: 0.4×10^8 cells, the third: 3.2×10^8 cells, and the fourth: 2.6×10^9 cells. Enrolment and infusion is complete	Bluestone, in preparation

UCB, umbilical cord blood; GVHD, graft versus host disease; BMT, bone marrow transplantation.

supporting both cell engraftment and the chance of inducing tolerance, such as transient host T cell depletion instituted following immunosuppressive treatments (120, 121).

As already discussed, the immunosuppression regimen for the ONE study includes the combination of CNI, tacrolimus together with prednisolone, and MMF. The main question that arises next is how this microenvironment will influence the Tregs *in vivo* following adoptive transfer.

In this regard, studies have shown that the use of CNIs during adoptive Treg therapy may have an indirect impact on the survival and suppressive ability of Tregs in view of the strong dependence of these cells on the exogenous supply of IL-2 (122, 123).

In agreement, studies in animal models have reported that CNI treatment reduces FOXP3 expression in natural Tregs (123, 124), diminishes the frequencies of $CD4^+CD25^+FOXP3^+$ T cells (81), and fails to support the differentiation of the highly suppressive $CD4^+CD25^+CD27^+$ Treg subset upon alloantigen stimulation (125). Studies in humans confirmed the negative effect of this treatment on Tregs, suggesting that the continuous CNI therapy is linked with progressive decline in Treg numbers (126). However, despite these major direct drawbacks on Tregs, it may be worth considering that CNI therapy may be still used to set up a favorable environment before the Treg infusion or at sub-therapeutic doses in combination with other drugs during Treg therapy. In this regard, Wang and collaborators showed that kidney transplanted recipients treated with MMF and low-dose tacrolimus had an induction of $CD4^+CD25^+FOXP3^+$ Tregs that could expand in the periphery and accumulate in the allograft. Additionally, the *in vitro* analysis of these cells confirmed the maintenance of their suppressive function (93).

On the other hand, the effects of MMF on Tregs have not been extensively analyzed and the few results reported are controversial. Data in literature support the idea that the influence of this drug on cell division may alter the expansion of antigen-specific Tregs and prevent the settlement of a long-term tolerance. In line with this notion, MMF administration in a murine model significantly inhibited the expansion of OVA-specific $CD4^+CD25^+Foxp3^+$ Tregs after OVA immunization (127). Other studies, however, propose that MMF has no effect on Tregs or may facilitate the induction of a more tolerogenic environment (123).

Corticosteroids, such as dexamethasone and prednisolone, have been used for decades as basis for the treatment of inflammatory diseases and in patients post-organ transplantation. They regulate a wide spectrum of physiological processes and control not only inflammation but also carbohydrate and protein metabolism, fetal development, and behavior. For these reasons, despite their therapeutic efficacy, there are major drawbacks associated with the persistent use of glucocorticoids, such as osteoporosis and diabetes (128). However, in respect to the impact of these treatments on Treg therapy, many authors described positive effects of steroids on the maturation and expansion of Tregs. Glucocorticoids have been suggested to amplify the IL-2-dependent expansion of $FOXP3^+CD4^+CD25^+$ T cells *in vivo* (129), increase FOXP3 expression by Tregs in patients affected by asthma (130), and restore the impaired suppressive function of Tregs in patients with relapsing multiple sclerosis (131). Furthermore, steroids may affect the inflammatory environment negatively controlling both Th1- and Th17-polarization in mice and humans (132–134).

This highlights the importance of strategies to tailor immunosuppressive therapy to ensure the *in vivo* survival of the injected Tregs or enhance their longevity *in vivo*. In this regard, the clinical protocol for ThRIL is based on a Treg-supportive immunosuppressive regimen including the use anti-thymocyte globulin (ATG), to induce lymphopenia with a preferential preservation of Tregs (135). Additionally, to limit memory T cell expansion post-ATG induction, patients are started on tacrolimus and prednisolone. One month prior to Treg infusion, in parallel with low-dose tacrolimus, the patients are given rapamycin, to promote selective Treg expansion *in vivo* (136). The intention behind this protocol: to create a tolerogenic milieu thus maximizing the potential efficacy of the exogenously administered Tregs through prolongation of their *in vivo* survival. It is also reassuring that these cells will be injected in a "Treg nurturing" environment, centered on the inclusion of rapamycin.

Thus, tailoring the immunosuppressive regimen along with the administration of *ex vivo*-expanded Tregs may potentially maintain post-liver transplant tolerance, accomplishing the ultimate aim of Treg immunotherapy trials in this setting.

Stability and Longevity of the Injected Cells and Visualization *In Vivo*

For Treg cellular therapy to be a viable therapeutic avenue, two key factors need to be addressed. The first being that, following injection, the Tregs are stable in the graft and draining lymph nodes, irrespective of the local inflammatory environment following transplantation, and second, whether these cells are either long-lived or able to impart their tolerance to the host immune system.

As the function of Tregs is highly dependent on the constitutively high expression of FOXP3 (137), many groups have sought to find ways to stabilize its expression. As discussed earlier, epigenetic modification of the *FOXP3* locus has a major role in controlling *FOXP3* transcription, with demethylation of key regions correlated with suppressive function and lineage stability (138). In this regard, *in vitro* treatment with demethylating agents, such as azacytidine, have shown to promote the stability of FOXP3 expression in Tregs, resulting in the potent ability of these treated cells to protect from GvHD (139). In addition, a recent Phase I trial has shown that patients with acute myeloid leukemia, treated with azacytidine immediately after allogeneic stem cell transplantation, had a higher proportion of Tregs as compared to time-matched controls (140).

FOXP3 levels are not only regulated through transcriptional control but also through post-translational modifications. In the context of transplantation, most work has focused on acetylation of lysine residues, which is known to stabilize the FOXP3 protein (141, 142). It has been shown that inhibiting deacetylation with histone deacetylase (HDAC) inhibitors or genetically removing Sirtuin-1, a histone and protein deacetylase, leads to an improvement in Treg function and stability, ultimately leading to improved allograft survival (143). Thus, future directions of adoptive Treg cell therapy will necessitate further understanding

of factors that cause Tregs to lose FOXP3 expression and ways to stabilize its expression.

The question of how long transferred Tregs survive *in vivo* is also of critical importance. It is understood that in order to establish long-term dominant tolerance, adoptively transferred Tregs must either survive and expand in the recipient, or be able to induce a tolerogenic phenotype on other T cells, a process known as infectious tolerance (144). It has been shown that some subpopulation of Tregs, such as those producing soluble factors, such as TGF-β (145), IL-10, and IL-35 (146), and the ongoing presence of recipient "infected" Tregs, are required to prevent allograft rejection (147, 148).

In the recent clinical trial of Treg therapy in hematopoietic stem cell transplantation, the transferred cells were no longer detected in the circulation after 2 weeks (56). Moreover, in the pediatric trial of Treg therapy in Type-I diabetes, infusion of 30×10^6/kg polyclonally expanded Tregs resulted in doubling of the percentage of circulating Tregs and a trend of increase at 2 weeks (118). In these trials, it is not known whether the cells migrated to tissues or died. In this regard, we have recently used single photon emission computed tomography to image adoptively transferred Tregs in mice and reported that 24 h after intravenous injection, the cells were primarily localized in the spleen (149).

Therefore, to maximize the efficacy of Treg therapy, efforts will need to focus on finding ways to support the *in vivo* survival, engraftment, and function of the infused Tregs. Since Tregs depend on exogenous IL-2 for survival, a suggested approach has been to use low-dose IL-2, which lacks the toxicity and immunostimulatory effects of the higher IL-2 doses used to treat cancer patients (150). This approach has recently shown to increase the number of Tregs in patients with chronic GvHD (151), supporting the notion that low-dose IL-2 may be an ideal adjuvant to adoptive Treg cell therapy, by promoting Treg expansion in an otherwise inflammatory setting.

The future will also see studies defining the trafficking patterns of infused Tregs *in vivo*. In this regard, in a recent clinical trial of Treg immunotherapy in Type-I diabetes conducted at UCSF, Tregs were labeled with deuterium and their relative homing and survival period was recorded *in vivo* (*Bluestone* et al. *unpublished data*). In parallel, micro-PET computed tomography fusion has been used clinically to track infused T cells in the body and has further been refined to focus on distinct T cell populations, in particular Tregs (152). While these technologies are relatively new, the information gleaned from their inclusion in clinical trial protocols of Treg cell therapy will be invaluable, allowing for virtual visualization of these cells *in vivo*.

The future of cell therapy is also moving in such a way through cellular engineering, introducing concepts of traceable markers, tunable TCRs, chemotactic receptors to synthetic ligands, and drug inducible suicidal enzymes (153). These designer features would not only allow for the monitoring of infused Tregs, while also controlling their activities and trafficking patterns, but also for elimination if and when they become pathogenic (154, 155). Nonetheless, further advances in gene therapy would be required for these approaches to move forward, with licensing issues posing their own challenges and hurdles.

Dose of Regulatory T Cells, Number Of Injections, and Monitoring Outcomes

As in the ThRIL trial, the first trials of Treg therapy in solid-organ transplantation have started with a dose escalation study to assess the safety and tolerability of Tregs at various doses. It is anticipated that high Treg doses are needed for tolerance induction in view of pre-clinical studies in mouse models of transplantation where a high ratio of Tregs to Teffectors, in the order of 1:1–1:2, i.e., 33–50% of Tregs, is needed to prevent transplant rejection (29, 156). Moreover, it has been suggested that, combined with ATG induction, a single infusion of $3–5 \times 10^9$ Tregs can effectively increase Treg percentage to more than 33% (157). One caveat is the use of antigen-specific Tregs, where studies have shown that lower numbers are needed to achieve the same functional efficacy as larger numbers of polyclonal Tregs (158, 159). Irrespectively, producing such large numbers of Tregs remains technically challenging, especially in view of studies showing a loss of FOXP3 expression after several rounds of stimulation. In this regard, more research is needed to understand Treg commitment and epigenetic regulation of FOXP3 expression so that the mechanisms can be harnessed to stabilize the Tregs.

Another point of consideration is if a single injection of Tregs is sufficient or whether multiple injections are required. This may be determined in larger Phase II efficacy studies, where patient outcomes should also be measured and an in-depth patient monitoring system planned. In this regard, molecular diagnostic tools can be utilized to assess a broad panel of biomarkers, associated with operational tolerance, to serve as surrogate end-points of efficacy (160–162).

In this regard, high-throughput, highly sensitive flow cytometric analysis can also be used to determine if the number of Tregs in the peripheral blood of recipients have increased or relatively quantify the composition of the T cell compartment following the intervention (163). Furthermore, the cytokine profile secretion capacity of these cells can be analyzed and thus their plasticity evaluated. Investigations using the complementarity-determining region 3 (CDR3) length distribution analysis can be used to explore the diversity of the TCR, in view of studies suggesting that the TCR repertoire might be a good predictor of graft outcome. In this regard, it has been suggested that the majority of kidney transplant patients with chronic rejection have an accumulation of oligo or monoclonal Vβ expansions while operationally tolerant recipients have a TCR repertoire like that of healthy individuals (164).

As such, a comprehensive immune monitoring plan of patients should be an integral part of a Treg therapy trial in order to gain mechanistic insight on the Treg function in patients. In addition, success in defining optimal ways of measuring tolerance would set the scene for subsequent trials in which accelerated drug minimization is the principal aim.

Anticipated Cost and the Future

At present, the cost to manufacture a single "personalized" injection of Tregs in the CRF is over £20,000 in the UK. The data soon emerging on the safety of these cells in the setting of

transplantation will provide the basis for progression to a larger Phase II/III study. The future progression of the cell therapy program will also see efforts focused on the optimization of the process development and potential commercialization of the cell-based therapies, through collaborations with industry and other organizations. It is anticipated that the future optimization of the manufacturing process for larger scale trials and commercialization would reduce the costs, making this modality of treatment broadly available and applicable in other disease settings.

Conclusion

We are now entering an exciting era in the study of immunological tolerance. Several cellular and molecular strategies of tolerance induction have been developed in non-human transplant models that have shown considerable promise and are just now appearing in clinical trials. As such, the recent progress in Treg biology and the successes in the clinical grade manufacture of these cells has seen the start of clinical trials of Treg therapy in solid-organ transplantation. Such trials will provide the basis for progression to a larger Phase II/III study with a comprehensive patient immune monitoring plan and the use of biomarkers that can predict the successful induction of immune tolerance, allowing for the safe minimization/withdrawal of immunosuppression. With this all said, it is no secret that the panacea of immunological tolerance in transplantation is now ordained as we take steps ever closer to its fulfilment.

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References

1. Meier-Kriesche HU, Schold JD, Kaplan B. Long-term renal allograft survival: have we made significant progress or is it time to rethink our analytic and therapeutic strategies? *Am J Transplant* (2004) **4**(8):1289–95. doi:10.1111/j.1600-6143.2004.00515.x
2. Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology* (1970) **18**(5):723–37.
3. Safinia N, Sagoo P, Lechler R, Lombardi G. Adoptive regulatory T cell therapy: challenges in clinical transplantation. *Curr Opin Organ Transplant* (2010) **15**(4):427–34. doi:10.1097/MOT.0b013e32833bfadc
4. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* (1995) **155**(3):1151–64.
5. Fehervari Z, Sakaguchi S. CD4+ tregs and immune control. *J Clin Invest* (2004) **114**(9):1209–17. doi:10.1172/JCI200423395
6. Povoleri GA, Scotta C, Nova-Lamperti EA, John S, Lombardi G, Afzali B. Thymic versus induced regulatory T cells – who regulates the regulators? *Front Immunol* (2013) **4**:169. doi:10.3389/fimmu.2013.00169
7. Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* (1994) **265**(5176):1237–40. doi:10.1126/science.7520605
8. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* (1997) **389**(6652):737–42. doi:10.1038/39614
9. Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, et al. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med* (2002) **195**(5):603–16. doi:10.1084/jem.20011629
10. Levings MK, Sangregorio R, Galbiati F, Squadrone S, de Waal Malefyt R, Roncarolo MG. IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *J Immunol* (2001) **166**(9):5530–9. doi:10.4049/jimmunol.166.9.5530
11. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* (2007) **204**(8):1757–64. doi:10.1084/jem.20070590
12. Cobbold SP, Waldmann H. Regulatory cells and transplantation tolerance. *Cold Spring Harb Perspect Med* (2013) **3**:a015545. doi:10.1101/cshperspect.a015545
13. Yadav M, Louvet C, Davini D, Gardner JM, Martinez-Llordella M, Bailey-Bucktrout S, et al. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. *J Exp Med* (2012) **209**(10):1713–22. doi:10.1084/jem.20120822
14. Weiss JM, Bilate AM, Gobert M, Ding Y, Curotto de Lafaille MA, Parkhurst CN, et al. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J Exp Med* (2012) **209**(10):1723–42. doi:10.1084/jem.20120914
15. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* (2003) **299**(5609):1057–61. doi:10.1126/science.1079490
16. Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* (2007) **445**(7129):766–70. doi:10.1038/nature05479
17. Huehn J, Polansky JK, Hamann A. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol* (2009) **9**(2):83–9. doi:10.1038/nri2474
18. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with Foxp3 and suppressive function of human CD4+ T reg cells. *J Exp Med* (2006) **203**(7):1701–11. doi:10.1084/jem.20060772
19. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the Foxp3 transcription factor. *Immunity* (2009) **30**(6):899–911. doi:10.1016/j.immuni.2009.03.019
20. Booth NJ, McQuaid AJ, Sobande T, Kissane S, Agius E, Jackson SE, et al. Different proliferative potential and migratory characteristics of human CD4+ regulatory T cells that express either CD45RA or CD45RO. *J Immunol* (2010) **184**(8):4317–26. doi:10.4049/jimmunol.0903781
21. Afzali B, Mitchell PJ, Edozie FC, Povoleri GA, Dowson SE, Demandt L, et al. CD161 expression characterizes a subpopulation of human regulatory T cells that produces IL-17 in a STAT3-dependent manner. *Eur J Immunol* (2013) **43**(8):2043–54. doi:10.1002/eji.201243296
22. Pesenacker AM, Bending D, Ursu S, Wu Q, Nistala K, Wedderburn LR. CD161 defines the subset of FoxP3+ T cells capable of producing proinflammatory cytokines. *Blood* (2013) **121**(14):2647–58. doi:10.1182/blood-2012-08-443473

23. Schmetterer KG, Neunkirchner A, Pickl WF. Naturally occurring regulatory T cells: markers, mechanisms, and manipulation. *FASEB J* (2012) **26**(6):2253–76. doi:10.1096/fj.11-193672
24. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* (2000) **192**(2):295–302. doi:10.1084/jem.192.2.295
25. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* (2011) **332**(6029):600–3. doi:10.1126/science.1202947
26. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* (2000) **192**(2):303–10. doi:10.1084/jem.192.2.303
27. Fahlen L, Read S, Gorelik L, Hurst SD, Coffman RL, Flavell RA, et al. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *J Exp Med* (2005) **201**(5):737–46. doi:10.1084/jem.20040685
28. Powrie F, Carlino J, Leach MW, Mauze S, Coffman RL. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. *J Exp Med* (1996) **183**(6):2669–74. doi:10.1084/jem.183.6.2669
29. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol* (2001) **166**(6):3789–96. doi:10.4049/jimmunol.166.6.3789
30. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* (2007) **450**(7169):566–9. doi:10.1038/nature06306
31. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* (2005) **174**(4):1783–6. doi:10.4049/jimmunol.174.4.1783
32. Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP, Ley TJ. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* (2004) **104**(9):2840–8. doi:10.1182/blood-2004-03-0859
33. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* (2007) **204**(6):1257–65. doi:10.1084/jem.20062512
34. Fletcher JM, Lonergan R, Costelloe L, Kinsella K, Moran B, O'Farrelly C, et al. CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. *J Immunol* (2009) **183**(11):7602–10. doi:10.4049/jimmunol.0901881
35. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol* (2003) **3**(3):199–210. doi:10.1038/nri1027
36. Hanash AM, Levy RB. Donor CD4+CD25+ T cells promote engraftment and tolerance following MHC-mismatched hematopoietic cell transplantation. *Blood* (2005) **105**(4):1828–36. doi:10.1182/blood-2004-08-3213
37. Joffre O, Gorsse N, Romagnoli P, Hudrisier D, van Meerwijk JP. Induction of antigen-specific tolerance to bone marrow allografts with CD4+CD25+ T lymphocytes. *Blood* (2004) **103**(11):4216–21. doi:10.1182/blood-2004-01-0005
38. Sanchez-Fueyo A, Weber M, Domenig C, Strom TB, Zheng XX. Tracking the immunoregulatory mechanisms active during allograft tolerance. *J Immunol* (2002) **168**(5):2274–81. doi:10.4049/jimmunol.168.5.2274
39. Dijke IE, Weimar W, Baan CC. Regulatory T cells after organ transplantation: where does their action take place? *Hum Immunol* (2008) **69**(7):389–98. doi:10.1016/j.humimm.2008.05.006
40. Auchincloss H Jr, Lee R, Shea S, Markowitz JS, Grusby MJ, Glimcher LH. The role of "indirect" recognition in initiating rejection of skin grafts from major histocompatibility complex class II-deficient mice. *Proc Natl Acad Sci U S A* (1993) **90**(8):3373–7. doi:10.1073/pnas.90.8.3373
41. Wise MP, Bemelman F, Cobbold SP, Waldmann H. Linked suppression of skin graft rejection can operate through indirect recognition. *J Immunol* (1998) **161**(11):5813–6.
42. Quezada SA, Fuller B, Jarvinen LZ, Gonzalez M, Blazar BR, Rudensky AY, et al. Mechanisms of donor-specific transfusion tolerance: preemptive induction of clonal T-cell exhaustion via indirect presentation. *Blood* (2003) **102**(5):1920–6. doi:10.1182/blood-2003-02-0586
43. Spadafora-Ferreira M, Caldas C, Fae KC, Marrero I, Monteiro SM, Lin-Wang HT, et al. CD4+CD25+Foxp3+ indirect alloreactive T cells from renal transplant patients suppress both the direct and indirect pathways of allorecognition. *Scand J Immunol* (2007) **66**(2–3):352–61. doi:10.1111/j.1365-3083.2007.01976.x
44. Yamada A, Chandraker A, Laufer TM, Gerth AJ, Sayegh MH, Auchincloss H Jr. Recipient MHC class II expression is required to achieve long-term survival of murine cardiac allografts after costimulatory blockade. *J Immunol* (2001) **167**(10):5522–6. doi:10.4049/jimmunol.167.10.5522
45. Golshayan D, Jiang S, Tsang J, Garin MI, Mottet C, Lechler RI. In vitro-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance. *Blood* (2007) **109**(2):827–35. doi:10.1182/blood-2006-05-025460
46. Tsang JY, Tanriver Y, Jiang S, Leung E, Ratnasothy K, Lombardi G, et al. Indefinite mouse heart allograft survival in recipient treated with CD4(+) CD25(+) regulatory T cells with indirect allospecificity and short term immunosuppression. *Transpl Immunol* (2009) **21**(4):203–9. doi:10.1016/j.trim.2009.05.003
47. Joffre O, Santolaria T, Calise D, Al Saati T, Hudrisier D, Romagnoli P, et al. Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. *Nat Med* (2008) **14**(1):88–92. doi:10.1038/nm1688
48. Nadig SN, Wieckiewicz J, Wu DC, Warnecke G, Zhang W, Luo S, et al. In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. *Nat Med* (2010) **16**(7):809–13. doi:10.1038/nm.2154
49. Xiao F, Ma L, Zhao M, Huang G, Mirenda V, Dorling A, et al. Ex vivo expanded human regulatory T cells delay islet allograft rejection via inhibiting islet-derived monocyte chemoattractant protein-1 production in CD34+ stem cells-reconstituted NOD-scid IL2rgammanull mice. *PLoS One* (2014) **9**(3):e90387. doi:10.1371/journal.pone.0090387
50. Issa F, Hester J, Goto R, Nadig SN, Goodacre TE, Wood K. Ex vivo-expanded human regulatory T cells prevent the rejection of skin allografts in a humanized mouse model. *Transplantation* (2010) **90**(12):1321–7. doi:10.1097/TP.0b013e3181ff8772
51. Wu DC, Hester J, Nadig SN, Zhang W, Trzonkowski P, Gray D, et al. Ex vivo expanded human regulatory T cells can prolong survival of a human islet allograft in a humanized mouse model. *Transplantation* (2013) **96**(8):707–16. doi:10.1097/TP.0b013e31829fa271
52. Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. *Nat Rev Immunol* (2007) **7**(2):118–30. doi:10.1038/nri2017
53. Sagoo P, Ali N, Garg G, Nestle FO, Lechler RI, Lombardi G. Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells. *Sci Transl Med* (2011) **3**(83):83ra42. doi:10.1126/scitranslmed.3002076
54. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* (2003) **9**(9):1144–50. doi:10.1038/nm915
55. Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, et al. Recipient-type specific CD4+CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J Clin Invest* (2003) **112**(11):1688–96. doi:10.1172/JCI17702
56. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* (2011) **117**(3):1061–70. doi:10.1182/blood-2010-07-293795
57. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* (2011) **117**(14):3921–8. doi:10.1182/blood-2010-10-311894
58. Trzonkowski P, Bieniaszewska M, Juscinska J, Dobyszuk A, Krzystyniak A, Marek N, et al. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. *Clin Immunol* (2009) **133**(1):22–6. doi:10.1016/j.clim.2009.06.001
59. Peters JH, Preijers FW, Woestenenk R, Hilbrands LB, Koenen HJ, Joosten I. Clinical grade Treg: GMP isolation, improvement of purity by CD127

depletion, Treg expansion, and Treg cryopreservation. *PLoS One* (2008) **3**(9):e3161. doi:10.1371/journal.pone.0003161
60. Putnam AL, Brusko TM, Lee MR, Liu W, Szot GL, Ghosh T, et al. Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes* (2009) **58**(3):652–62. doi:10.2337/db08-1168
61. Hoffmann P, Eder R, Boeld TJ, Doser K, Piseshka B, Andreesen R, et al. Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. *Blood* (2006) **108**(13):4260–7. doi:10.1182/blood-2006-06-027409
62. Hoffmann P, Boeld TJ, Eder R, Huehn J, Floess S, Wieczorek G, et al. Loss of FOXP3 expression in natural human CD4+CD25+ regulatory T cells upon repetitive in vitro stimulation. *Eur J Immunol* (2009) **39**(4):1088–97. doi:10.1002/eji.200838904
63. Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J, et al. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *Eur J Immunol* (2007) **37**(9):2378–89. doi:10.1002/eji.200737594
64. Polansky JK, Schreiber L, Thelemann C, Ludwig L, Kruger M, Baumgrass R, et al. Methylation matters: binding of Ets-1 to the demethylated Foxp3 gene contributes to the stabilization of Foxp3 expression in regulatory T cells. *J Mol Med* (2010) **88**(10):1029–40. doi:10.1007/s00109-010-0642-1
65. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* (2010) **463**(7282):808–12. doi:10.1038/nature08750
66. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* (2008) **29**(1):44–56. doi:10.1016/j.immuni.2008.05.007
67. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol* (2009) **10**(9):1000–7. doi:10.1038/ni.1774
68. Oldenhove G, Bouladoux N, Wohlfert EA, Hall JA, Chou D, Dos Santos L, et al. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity* (2009) **31**(5):772–86. doi:10.1016/j.immuni.2009.10.001
69. Sharma MD, Hou DY, Baban B, Koni PA, He Y, Chandler PR, et al. Reprogrammed foxp3(+) regulatory T cells provide essential help to support cross-presentation and CD8(+) T cell priming in naive mice. *Immunity* (2010) **33**(6):942–54. doi:10.1016/j.immuni.2010.11.022
70. Komatsu N, Mariotti-Ferrandiz ME, Wang Y, Malissen B, Waldmann H, Hori S. Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci U S A* (2009) **106**(6):1903–8. doi:10.1073/pnas.0811556106
71. Canavan JB, Scotta C, Vossenkamper A, Goldberg R, Elder MJ, Shoval I, et al. Developing in vitro expanded CD45RA+ regulatory T cells as an adoptive cell therapy for Crohn's disease. *Gut* (2015) 1–11. doi:10.1136/gutjnl-2014-306919
72. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* (2006) **203**(7):1693–700. doi:10.1084/jem.20060468
73. Hulspas R, Villa-Komaroff L, Koksal E, Etienne K, Rogers P, Tuttle M, et al. Purification of regulatory T cells with the use of a fully enclosed high-speed microfluidic system. *Cytotherapy* (2014) **16**(10):1384–9. doi:10.1016/j.jcyt.2014.05.016
74. Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* (2001) **193**(11):1295–302. doi:10.1084/jem.193.11.1295
75. Thomson AW, Turnquist HR, Raimondi G. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol* (2009) **9**(5):324–37. doi:10.1038/nri2546
76. Zeiser R, Leveson-Gower DB, Zambricki EA, Kambham N, Beilhack A, Loh J, et al. Differential impact of mammalian target of rapamycin inhibition on CD4+CD25+Foxp3+ regulatory T cells compared with conventional CD4+ T cells. *Blood* (2008) **111**(1):453–62. doi:10.1182/blood-2007-06-094482
77. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* (2005) **105**(12):4743–8. doi:10.1182/blood-2004-10-3932
78. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* (2009) **30**(6):832–44. doi:10.1016/j.immuni.2009.04.014
79. Hester J, Schiopu A, Nadig SN, Wood KJ. Low-dose rapamycin treatment increases the ability of human regulatory T cells to inhibit transplant arteriosclerosis in vivo. *Am J Transplant* (2012) **12**(8):2008–16. doi:10.1111/j.1600-6143.2012.04065.x
80. Noris M, Casiraghi F, Todeschini M, Cravedi P, Cugini D, Monteferrante G, et al. Regulatory T cells and T cell depletion: role of immunosuppressive drugs. *J Am Soc Nephrol* (2007) **18**(3):1007–18. doi:10.1681/ASN.2006101143
81. Segundo DS, Ruiz JC, Izquierdo M, Fernandez-Fresnedo G, Gomez-Alamillo C, Merino R, et al. Calcineurin inhibitors, but not rapamycin, reduce percentages of CD4+CD25+FOXP3+ regulatory T cells in renal transplant recipients. *Transplantation* (2006) **82**(4):550–7. doi:10.1097/01.tp.0000229473.95202.50
82. Basu S, Golovina T, Mikheeva T, June CH, Riley JL. Cutting edge: foxp3-mediated induction of pim 2 allows human T regulatory cells to preferentially expand in rapamycin. *J Immunol* (2008) **180**(9):5794–8. doi:10.4049/jimmunol.180.9.5794
83. Tresoldi E, Dell'albani I, Stabilini A, Jofra T, Valle A, Gagliani N, et al. Stability of human rapamycin-expanded CD4+CD25+ T regulatory cells. *Haematologica* (2011) **96**(9):1357–65. doi:10.3324/haematol.2011.041483
84. Golovina TN, Mikheeva T, Brusko TM, Blazar BR, Bluestone JA, Riley JL. Retinoic acid and rapamycin differentially affect and synergistically promote the ex vivo expansion of natural human T regulatory cells. *PLoS One* (2011) **6**(1):e15868. doi:10.1371/journal.pone.0015868
85. Scotta C, Esposito M, Fazekasova H, Fanelli G, Edozie FC, Ali N, et al. Differential effects of rapamycin and retinoic acid on expansion, stability and suppressive qualities of human CD4+CD25+Foxp3+ Treg subpopulations. *Haematologica* (2012). doi:10.3324/haematol.2012.074088
86. Abele-Ohl S, Leis M, Mahmoudian S, Weyand M, Stamminger T, Ensminger SM. Rag2-/- gamma-chain-/- mice as hosts for human vessel transplantation and allogeneic human leukocyte reconstitution. *Transpl Immunol* (2010) **23**(1–2):59–64. doi:10.1016/j.trim.2010.04.003
87. Rossetti M, Spreafico R, Saidin S, Chua C, Moshref M, Leong JY, et al. Ex vivo-expanded but not in vitro-induced human regulatory T cells are candidates for cell therapy in autoimmune diseases thanks to stable demethylation of the Foxp3 regulatory T cell-specific demethylated region. *J Immunol* (2015) **194**(1):113–24. doi:10.4049/jimmunol.1401145
88. Theodosiou M, Laudet V, Schubert M. From carrot to clinic: an overview of the retinoic acid signaling pathway. *Cell Mol Life Sci* (2010) **67**(9):1423–45. doi:10.1007/s00018-010-0268-z
89. Hall JA, Cannons JL, Grainger JR, Dos Santos LM, Hand TW, Naik S, et al. Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. *Immunity* (2011) **34**(3):435–47. doi:10.1016/j.immuni.2011.03.003
90. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* (2007) **317**(5835):256–60. doi:10.1126/science.1145697
91. Elias KM, Laurence A, Davidson TS, Stephens G, Kanno Y, Shevach EM, et al. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. *Blood* (2008) **111**(3):1013–20. doi:10.1182/blood-2007-06-096438
92. Mucida D, Pino-Lagos K, Kim G, Nowak E, Benson MJ, Kronenberg M, et al. Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naive T cells. *Immunity* (2009) **30**(4):471–2. doi:10.1016/j.immuni.2009.03.008
93. Wang Z, Shi B, Jin H, Xiao L, Chen Y, Qian Y. Low-dose of tacrolimus favors the induction of functional CD4(+)CD25(+)FoxP3(+) regulatory T cells in solid-organ transplantation. *Int Immunopharmacol* (2009) **9**(5):564–9. doi:10.1016/j.intimp.2009.01.029
94. Lu L, Lan Q, Li Z, Zhou X, Gu J, Li Q, et al. Critical role of all-trans retinoic acid in stabilizing human natural regulatory T cells under inflammatory conditions. *Proc Natl Acad Sci U S A* (2014) **111**(33):E3432–40. doi:10.1073/pnas.1408780111

95. Kang SG, Lim HW, Andrisani OM, Broxmeyer HE, Kim CH. Vitamin A metabolites induce gut-homing FoxP3+ regulatory T cells. *J Immunol* (2007) **179**(6):3724–33. doi:10.4049/jimmunol.179.6.3724
96. McGrane MM. Vitamin A regulation of gene expression: molecular mechanism of a prototype gene. *J Nutr Biochem* (2007) **18**(8):497–508. doi:10.1016/j.jnutbio.2006.10.006
97. Lu L, Ma J, Li Z, Lan Q, Chen M, Liu Y, et al. All-trans retinoic acid promotes TGF-beta-induced Tregs via histone modification but not DNA demethylation on Foxp3 gene locus. *PLoS One* (2011) **6**(9):e24590. doi:10.1371/journal.pone.0024590
98. Basu R, Whitley SK, Bhaumik S, Zindl CL, Schoeb TR, Benveniste EN, et al. IL-1 signaling modulates activation of STAT transcription factors to antagonize retinoic acid signaling and control the TH17 cell-iTreg cell balance. *Nat Immunol* (2015) **16**(3):286–95. doi:10.1038/ni.3099
99. Chambers ES, Nanzer AM, Richards DF, Ryanna K, Freeman AT, Timms PM, et al. Serum 25-dihydroxyvitamin D levels correlate with CD4(+)Foxp3(+) T-cell numbers in moderate/severe asthma. *J Allergy Clin Immunol* (2012) **130**(2):542–4. doi:10.1016/j.jaci.2012.04.022
100. Milliken SV, Wassall H, Lewis BJ, Logie J, Barker RN, Macdonald H, et al. Effects of ultraviolet light on human serum 25-hydroxyvitamin D and systemic immune function. *J Allergy Clin Immunol* (2012) **129**(6):1554–61. doi:10.1016/j.jaci.2012.03.001
101. Smolders J, Thewissen M, Peelen E, Menheere P, Tervaert JW, Damoiseaux J, et al. Vitamin D status is positively correlated with regulatory T cell function in patients with multiple sclerosis. *PLoS One* (2009) **4**(8):e6635. doi:10.1371/journal.pone.0006635
102. Urry Z, Chambers ES, Xystrakis E, Dimeloe S, Richards DF, Gabrysova L, et al. The role of 1alpha,25-dihydroxyvitamin D3 and cytokines in the promotion of distinct Foxp3+ and IL-10+ CD4+ T cells. *Eur J Immunol* (2012) **42**(10):2697–708. doi:10.1002/eji.201242370
103. Chambers ES, Suwannasaen D, Mann EH, Urry Z, Richards DF, Lertmemongkolchai G, et al. 1alpha,25-dihydroxyvitamin D3 in combination with transforming growth factor-beta increases the frequency of Foxp3(+) regulatory T cells through preferential expansion and usage of interleukin-2. *Immunology* (2014) **143**(1):52–60. doi:10.1111/imm.12289
104. Putnam AL, Safinia N, Medvec A, Laszkowska M, Wray M, Mintz MA, et al. Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am J Transplant* (2013) **13**(11):3010–20. doi:10.1111/ajt.12433
105. Peters JH, Hilbrands LB, Koenen HJ, Joosten I. Ex vivo generation of human alloantigen-specific regulatory T cells from CD4(pos)CD25(high) T cells for immunotherapy. *PLoS One* (2008) **3**(5):e2233. doi:10.1371/journal.pone.0002233
106. Chen LC, Delgado JC, Jensen PE, Chen X. Direct expansion of human allospecific FoxP3+CD4+ regulatory T cells with allogeneic B cells for therapeutic application. *J Immunol* (2009) **183**(6):4094–102. doi:10.4049/jimmunol.0901081
107. Tran GT, Hodgkinson SJ, Carter NM, Verma ND, Plain KM, Boyd R, et al. IL-5 promotes induction of antigen-specific CD4+CD25+ T regulatory cells that suppress autoimmunity. *Blood* (2012) **119**(19):4441–50. doi:10.1182/blood-2011-12-396101
108. Adachi M, Ishii H. Role of mitochondria in alcoholic liver injury. *Free Radic Biol Med* (2002) **32**(6):487–91. doi:10.1016/S0891-5849(02)00740-2
109. Tu W, Lau YL, Zheng J, Liu Y, Chan PL, Mao H, et al. Efficient generation of human alloantigen-specific CD4+ regulatory T cells from naive precursors by CD40-activated B cells. *Blood* (2008) **112**(6):2554–62. doi:10.1182/blood-2008-04-152041
110. Zheng J, Liu Y, Lau YL, Tu W. CD40-activated B cells are more potent than immature dendritic cells to induce and expand CD4(+) regulatory T cells. *Cell Mol Immunol* (2010) **7**(1):44–50. doi:10.1038/cmi.2009.103
111. Brusko TM, Koya RC, Zhu S, Lee MR, Putnam AL, McClymont SA, et al. Human antigen-specific regulatory T cells generated by T cell receptor gene transfer. *PLoS One* (2010) **5**(7):e11726. doi:10.1371/journal.pone.0011726
112. Jethwa H, Adami AA, Maher J. Use of gene-modified regulatory T-cells to control autoimmune and alloimmune pathology: is now the right time? *Clin Immunol* (2014) **150**(1):51–63. doi:10.1016/j.clim.2013.11.004
113. Fransson M, Piras E, Burman J, Nilsson B, Essand M, Lu B, et al. CAR/FoxP3-engineered T regulatory cells target the CNS and suppress EAE upon intranasal delivery. *J Neuroinflammation* (2012) **9**:112. doi:10.1186/1742-2094-9-112
114. Blat D, Zigmond E, Alteber Z, Waks T, Eshhar Z. Suppression of murine colitis and its associated cancer by carcinoembryonic antigen-specific regulatory T cells. *Mol Ther* (2014) **22**(5):1018–28. doi:10.1038/mt.2014.41
115. Jiang S, Camara N, Lombardi G, Lechler RI. Induction of allopeptide-specific human CD4+CD25+ regulatory T cells ex vivo. *Blood* (2003) **102**(6):2180–6. doi:10.1182/blood-2003-04-1164
116. Veerapathran A, Pidala J, Beato F, Yu XZ, Anasetti C. Ex vivo expansion of human tregs specific for alloantigens presented directly or indirectly. *Blood* (2011) **118**(20):5671–80. doi:10.1182/blood-2011-02-337097
117. Tsang JY, Tanriver Y, Jiang S, Xue SA, Ratnasothy K, Chen D, et al. Conferring indirect allospecificity on CD4+CD25+ Tregs by TCR gene transfer favors transplantation tolerance in mice. *J Clin Invest* (2008) **118**(11):3619–28. doi:10.1172/JCI33185
118. Marek-Trzonkowska N, Mysliwiec M, Dobyszuk A, Grabowska M, Derkowska I, Juscinska J, et al. Therapy of type 1 diabetes with CD4(+)CD25(high)CD127-regulatory T cells prolongs survival of pancreatic islets – results of one year follow-up. *Clin Immunol* (2014) **153**(1):23–30. doi:10.1016/j.clim.2014.03.016
119. Yamashita K, Goto R, Zaitsu M, Agatsu AN, Oura T, Watanabe M, et al. Induction of operational tolerance by cell therapy using donor Ag-pulsed tregs in living donorliver transplantation. *World Transplant Congress San Francisco* (2014) **98**:871–905. doi:10.1097/01.tp.0000452138.48155.71
120. Wells AD, Li XC, Li Y, Walsh MC, Zheng XX, Wu Z, et al. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med* (1999) **5**(11):1303–7. doi:10.1038/8466
121. Xia G, He J, Leventhal JR. Ex vivo-expanded natural CD4+CD25+ regulatory T cells synergize with host T-cell depletion to promote long-term survival of allografts. *Am J Transplant* (2008) **8**(2):298–306. doi:10.1111/j.1600-6143.2007.02088.x
122. Furtado GC, Curotto de Lafaille MA, Kutchukhidze N, Lafaille JJ. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J Exp Med* (2002) **196**(6):851–7. doi:10.1084/jem.20020190
123. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, et al. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* (2006) **108**(1):390–9. doi:10.1182/blood-2006-01-0329
124. Baan CC, van der Mast BJ, Klepper M, Mol WM, Peeters AM, Korevaar SS, et al. Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. *Transplantation* (2005) **80**(1):110–7. doi:10.1097/01.TP.0000164142.98167.4B
125. Coenen JJ, Koenen HJ, van Rijssen E, Hilbrands LB, Joosten I. Rapamycin, and not cyclosporin A, preserves the highly suppressive CD27+ subset of human CD4+CD25+ regulatory T cells. *Blood* (2006) **107**(3):1018–23. doi:10.1182/blood-2005-07-3032
126. Akimova T, Kamath BM, Goebel JW, Meyers KE, Rand EB, Hawkins A, et al. Differing effects of rapamycin or calcineurin inhibitor on T-regulatory cells in pediatric liver and kidney transplant recipients. *Am J Transplant* (2012) **12**(12):3449–61. doi:10.1111/j.1600-6143.2012.04269.x
127. Wu T, Zhang L, Xu K, Sun C, Lei T, Peng J, et al. Immunosuppressive drugs on inducing Ag-specific CD4(+)CD25(+)Foxp3(+) Treg cells during immune response in vivo. *Transpl Immunol* (2012) **27**(1):30–8. doi:10.1016/j.trim.2012.05.001
128. McDonough AK, Curtis JR, Saag KG. The epidemiology of glucocorticoid-associated adverse events. *Curr Opin Rheumatol* (2008) **20**(2):131–7. doi:10.1097/BOR.0b013e3282f51031
129. Chen X, Oppenheim JJ, Winkler-Pickett RT, Ortaldo JR, Howard OM. Glucocorticoid amplifies IL-2-dependent expansion of functional FoxP3(+) CD4(+)CD25(+) T regulatory cells in vivo and enhances their capacity to suppress EAE. *Eur J Immunol* (2006) **36**(8):2139–49. doi:10.1002/eji.200635873
130. Karagiannidis C, Akdis M, Holopainen P, Woolley NJ, Hense G, Ruckert B, et al. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. *J Allergy Clin Immunol* (2004) **114**(6):1425–33. doi:10.1016/j.jaci.2004.07.014
131. Xu L, Xu Z, Xu M. Glucocorticoid treatment restores the impaired suppressive function of regulatory T cells in patients with relapsing-remitting multiple sclerosis. *Clin Exp Immunol* (2009) **158**(1):26–30. doi:10.1111/j.1365-2249.2009.03987.x

132. Blotta MH, DeKruyff RH, Umetsu DT. Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in CD4+ lymphocytes. *J Immunol* (1997) **158**(12):5589–95.
133. Luther C, Adamopoulou E, Stoeckle C, Brucklacher-Waldert V, Rosenkranz D, Stoltze L, et al. Prednisolone treatment induces tolerogenic dendritic cells and a regulatory milieu in myasthenia gravis patients. *J Immunol* (2009) **183**(2):841–8. doi:10.4049/jimmunol.0802046
134. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* (2007) **8**(9):967–74. doi:10.1038/ni1488
135. Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N. A novel mechanism of action for anti-thymocyte globulin: induction of CD4+CD25+Foxp3+ regulatory T cells. *J Am Soc Nephrol* (2006) **17**(10):2844–53. doi:10.1681/ASN.2006050422
136. Strauss L, Whiteside TL, Knights A, Bergmann C, Knuth A, Zippelius A. Selective survival of naturally occurring human CD4+CD25+Foxp3+ regulatory T cells cultured with rapamycin. *J Immunol* (2007) **178**(1):320–9. doi:10.4049/jimmunol.178.1.320
137. Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R, et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* (2007) **19**(4):345–54. doi:10.1093/intimm/dxm014
138. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, et al. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol* (2007) **5**(2):e38. doi:10.1371/journal.pbio.0050038
139. Sanchez-Abarca LI, Gutierrez-Cosio S, Santamaria C, Caballero-Velazquez T, Blanco B, Herrero-Sanchez C, et al. Immunomodulatory effect of 5-azacytidine (5-azaC): potential role in the transplantation setting. *Blood* (2010) **115**(1):107–21. doi:10.1182/blood-2009-03-210393
140. Goodyear OC, Dennis M, Jilani NY, Loke J, Siddique S, Ryan G, et al. Azacitidine augments expansion of regulatory T cells after allogeneic stem cell transplantation in patients with acute myeloid leukemia (AML). *Blood* (2012) **119**(14):3361–9. doi:10.1182/blood-2011-09-377044
141. Kwon HS, Lim HW, Wu J, Schnolzer M, Verdin E, Ott M. Three novel acetylation sites in the Foxp3 transcription factor regulate the suppressive activity of regulatory T cells. *J Immunol* (2012) **188**(6):2712–21. doi:10.4049/jimmunol.1100903
142. Liu Y, Wang L, Han R, Beier UH, Hancock WW. Two lysines in the forkhead domain of foxp3 are key to T regulatory cell function. *PLoS One* (2012) **7**(1):e29035. doi:10.1371/journal.pone.0029035
143. Beier UH, Wang L, Bhatti TR, Liu Y, Han R, Ge G, et al. Sirtuin-1 targeting promotes Foxp3+ T-regulatory cell function and prolongs allograft survival. *Mol Cell Biol* (2011) **31**(5):1022–9. doi:10.1128/MCB.01206-10
144. Kendal AR, Waldmann H. Infectious tolerance: therapeutic potential. *Curr Opin Immunol* (2010) **22**(5):560–5. doi:10.1016/j.coi.2010.08.002
145. Andersson J, Tran DQ, Pesu M, Davidson TS, Ramsey H, O'Shea JJ, et al. CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. *J Exp Med* (2008) **205**(9):1975–81. doi:10.1084/jem.20080308
146. Chaturvedi V, Collison LW, Guy CS, Workman CJ, Vignali DA. Cutting edge: human regulatory T cells require IL-35 to mediate suppression and infectious tolerance. *J Immunol* (2011) **186**(12):6661–6. doi:10.4049/jimmunol.1100315
147. Kendal AR, Chen Y, Regateiro FS, Ma J, Adams E, Cobbold SP, et al. Sustained suppression by Foxp3+ regulatory T cells is vital for infectious transplantation tolerance. *J Exp Med* (2011) **208**(10):2043–53. doi:10.1084/jem.20110767
148. Gagliani N, Jofra T, Valle A, Stabilini A, Morsiani C, Gregori S, et al. Transplant tolerance to pancreatic islets is initiated in the graft and sustained in the spleen. *Am J Transplant* (2013) **13**(8):1963–75. doi:10.1111/ajt.12333
149. Sharif-Paghaleh E, Sunassee K, Tavare R, Ratnasothy K, Koers A, Ali N, et al. In vivo SPECT reporter gene imaging of regulatory T cells. *PLoS One* (2011) **6**(10):e25857. doi:10.1371/journal.pone.0025857
150. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat Rev Immunol* (2012) **12**(3):180–90. doi:10.1038/nri3156
151. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP III, et al. Interleukin-2 and regulatory T cells in graft-versus-host disease. *N Engl J Med* (2011) **365**(22):2055–66. doi:10.1056/NEJMoa1108188
152. Ribas A, Koya RC. Adoptive cell transfer of T-cell receptor-engineered lymphocytes: lessons from recent modeling. *Future Oncol* (2010) **6**(11):1671–3. doi:10.2217/fon.10.137
153. Lim WA. Designing customized cell signalling circuits. *Nat Rev Mol cell Biol* (2010) **11**(6):393–403. doi:10.1038/nrm2904
154. Guillot-Delost M, Cherai M, Hamel Y, Rosenzwajg M, Baillou C, Simonin G, et al. Clinical-grade preparation of human natural regulatory T-cells encoding the thymidine kinase suicide gene as a safety gene. *J Gene Med* (2008) **10**(8):834–46. doi:10.1002/jgm.1220
155. Sato T, Neschadim A, Konrad M, Fowler DH, Lavie A, Medin JA. Engineered human tmpk/AZT as a novel enzyme/prodrug axis for suicide gene therapy. *Mol Ther* (2007) **15**(5):962–70. doi:10.1038/mt.sj.6300122
156. Graca L, Thompson S, Lin CY, Adams E, Cobbold SP, Waldmann H. Both CD4(+)CD25(+) and CD4(+)CD25(-) regulatory cells mediate dominant transplantation tolerance. *J Immunol* (2002) **168**(11):5558–65. doi:10.4049/jimmunol.168.11.5558
157. Tang Q, Lee K. Regulatory T-cell therapy for transplantation: how many cells do we need? *Curr Opin Organ Transplant* (2012) **17**(4):349–54. doi:10.1097/MOT.0b013e328355a992
158. Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, et al. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* (2004) **199**(11):1455–65. doi:10.1084/jem.20040139
159. Tarbell KV, Yamazaki S, Olson K, Toy P, Steinman RM. CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. *J Exp Med* (2004) **199**(11):1467–77. doi:10.1084/jem.20040180
160. Brouard S, Mansfield E, Braud C, Li L, Giral M, Hsieh SC, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci U S A* (2007) **104**(39):15448–53. doi:10.1073/pnas.0705834104
161. Martinez-Llordella M, Lozano JJ, Puig-Pey I, Orlando G, Tisone G, Lerut J, et al. Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. *J Clin Invest* (2008) **118**(8):2845–57. doi:10.1172/JCI35342
162. Sagoo P, Perucha E, Sawitzki B, Tomiuk S, Stephens DA, Miqueu P, et al. Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. *J Clin Invest* (2010) **120**(6):1848–61. doi:10.1172/JCI39922
163. Hoffmann J, Fiser K, Weaver J, Dimmick I, Loeher M, Pircher H, et al. High-throughput 13-parameter immunophenotyping identifies shifts in the circulating T-cell compartment following reperfusion in patients with acute myocardial infarction. *PLoS One* (2012) **7**(10):e47155. doi:10.1371/journal.pone.0047155
164. Miqueu P, Degauque N, Guillet M, Giral M, Ruiz C, Pallier A, et al. Analysis of the peripheral T-cell repertoire in kidney transplant patients. *Eur J Immunol* (2010) **40**(11):3280–90. doi:10.1002/eji.201040301

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Clinical Outlook for Type-1 and FOXP3$^+$ T Regulatory Cell-Based Therapy

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T regulatory cells (Tregs) are subsets of T lymphocytes specialized in modulating antigen-specific immune responses *in vivo*. Hence, Tregs represent an ideal therapeutic tool to control detrimental immune reactions. Based on solid pre-clinical results, investigators started testing the safety and efficacy of Treg-based therapies in humans. Despite promising results, a number of issues remain to be solved. We will discuss the results obtained from clinical trials and the challenges and risks we are facing in the further development of Treg-based therapies.

Keywords: T regulatory cells, T regulatory type 1 cells, tolerance, Treg-based therapy, IL-10, FOXP3, gene transfer

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INTRODUCTION

T regulatory cells (Tregs) are a component of the immune system involved in modulating immune reactions and in inducing tolerance. Due to their potential as immune modulators, therapeutic application of Tregs to control undesirable immune responses and to promote tolerance has become an active field of investigation (1). Over the years, several types of Tregs have been identified, and the forkhead box P3 (FOXP3)-expressing Tregs (FOXP3$^+$ Tregs) (2) and the T regulatory type 1 (Tr1) cells (3) are the best characterized (**Figure 1**).

FOXP3$^+$ Tregs can be either thymus-derived (tTregs), or induced in the periphery (pTregs) (4, 5). Regardless of their origin, both subsets are characterized by constitutive expression of the IL-2Rα-chain (CD25), in the absence of the IL-7Rα-chain (CD127), and of FOXP3 (6), making the two subsets indistinguishable based on their phenotype. High expression of Helios has been identified in FOXP3$^+$ Tregs (7), and suggested to be specific for tTregs (8). However, this notion was later challenged by the demonstration that Helios is also expressed by non-tTregs (9, 10). To date, the most reliable feature unambiguously identifying tTregs is the epigenetic remodeling of a specific region in the *FOXP3* locus, indicated as Treg-specific-demethylated-region (TSDR) (11). A more comprehensive CpG hypomethylation pattern of tTregs including several Treg-related genes has been described (12).

In addition to CD25, along the years, the expression of several molecules, i.e., CTLA-4 (13), GITR (14), CD39 (15), Galectin 10 (16), latency-associated-peptide (LAP) (17), and glycoproteinA-repetitions-predominant (GARP) (18) has been attributed to human FOXP3$^+$ Tregs. The expression of the above-mentioned molecules is not exclusive to FOXP3$^+$ Tregs, since they are often shared with activated conventional T cells.

CTLA-4, GITR, and CD39 are specifically associated with FOXP3$^+$ Treg suppressive function, which is primarily dependent on contact with target cells. Additional mechanisms of suppression have been described for FOXP3$^+$ Tregs, including release of IL-10 (19), TGF-β (20, 21), and IL-35

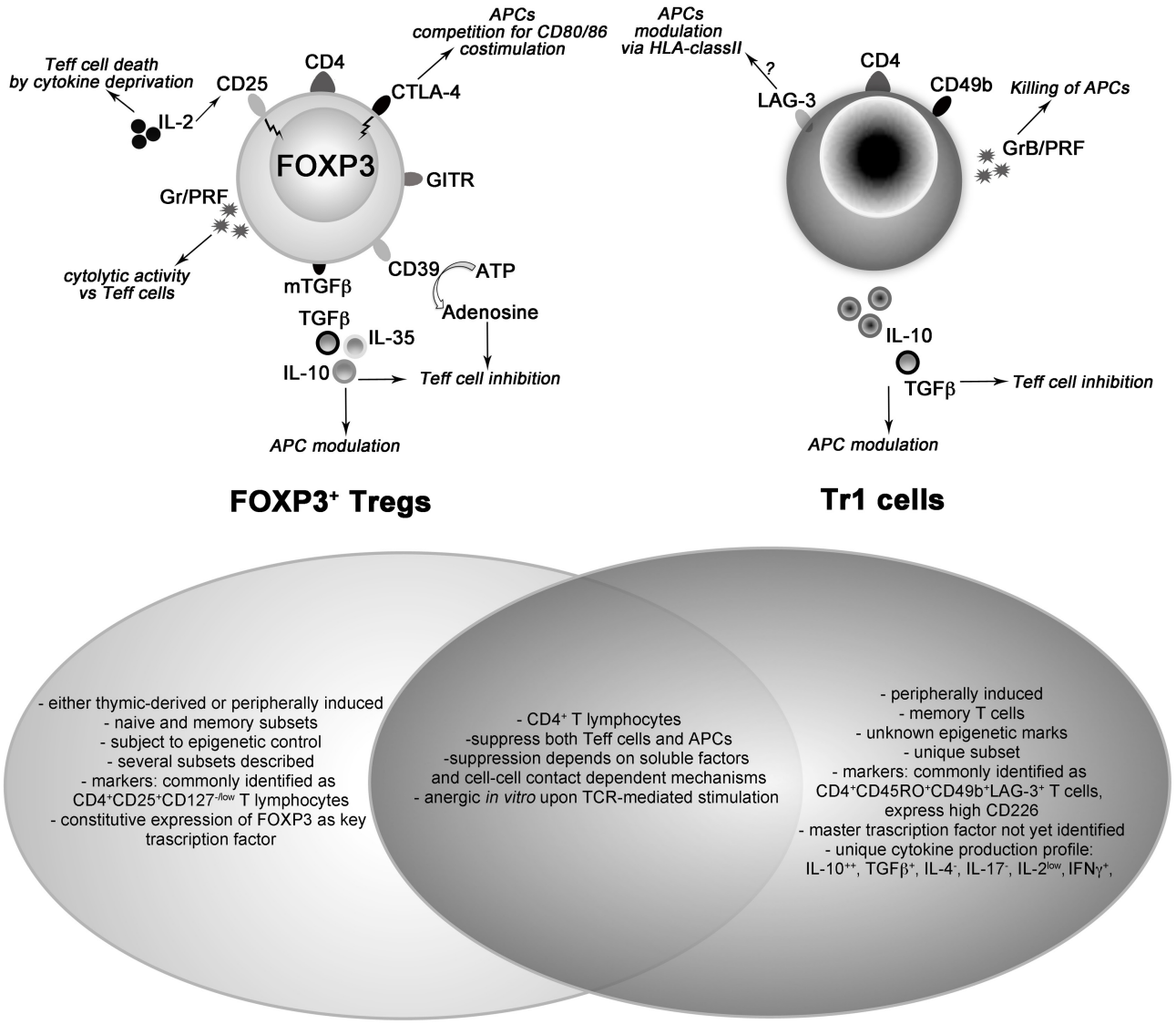

FIGURE 1 | Schematic representation of cell surface and intracytoplasmic markers and mechanisms of action characterizing FOXP3-expressing (left cartoon) and type 1 (right cartoon) T regulatory cells. The shared and unique features of both cell types are listed in the frames. Ag, antigen; APC, antigen-presenting cell; ATP, adenosine triphosphate; CTLA-4, cytotoxic T-lymphocyte antigen 4; FOXP3, forkhead box protein 3; GITR, glucocorticoid-induced TNFR family related gene; Gr; Granzymes; LAG-3, lymphocyte-activation gene 3; PRF, perforin; Teff, effector T cell; TGFβ, transforming growth factor β; Tr1, type 1 T regulatory cell.

(22), direct killing of T effector (Teff) cells through the granzyme/perforin axis (23), modulation of antigen-presenting cells (APCs) stimulatory capacity *via* CTLA-4 (24), cytokine deprivation (25), and generation of immunosuppressive metabolites, such as extracellular adenosine (26) and intracellular cAMP (27). The variety of phenotypes and weapons discovered led from the original idea of FOXP3$^+$ Tregs as homogeneous population to the modern view of a heterogeneous pool, including several specialized subtypes characterized by expression of specific cell surface markers such as ICOS (19), HLA-DR (28, 29), and CD45 isoforms (30, 31).

Tr1 cells are memory T lymphocytes expressing CD49b and LAG-3 (32). Tr1 cells, upon activation, secrete high levels of IL-10 and TGF-β, variable amounts of IL-5, GM-CSF, and IFN-γ, and minimal amounts of IL-2, IL-4, and IL-17 (3, 33, 34). Tr1 cells express CTLA-4, (35, 36), PD-1 (36), and ICOS (37). Similar to FOXP3$^+$ Tregs, Tr1 cells can express CD39 and CD73 [Ref. (38–41) and (Gregori et al. unpublished data)]. Tr1 cells do not constitutively express FOXP3 (42), thus they are distinct from both tTregs and pTregs; however, upon activation, Tr1 cells can transiently up-regulate FOXP3, but its expression never reaches the levels of FOXP3$^+$ Tregs (33, 43–45).

The main mechanism by which Tr1 cells control immune responses is the secretion of IL-10 and TGF-β. Importantly, to exert their suppressive function, Tr1 cells need to be activated *via* their TCR, but, once activated, they can mediate bystander suppressive activity against other antigen(Ag)s (3, 33). IL-10

and TGF-β directly inhibit T-cell responses by suppressing IL-2 and IFN-γ production and T-cell proliferation, and indirectly act on APCs by down-modulating costimulatory molecules, HLA-class-II, and pro-inflammatory cytokine production (34). In addition to the cytokine-mediated suppression, Tr1 cells inhibit T-cell responses by killing myeloid APCs *via* granzyme B (46). Tr1 cell-mediated cytotoxicity of myeloid APCs requires stable adhesion with target cells and activation *via* HLA-class-I molecules and CD112/CD155 expressed on target cells (46). New evidence suggests that Tr1 cells use additional modes of immune regulation to achieve tolerance: they can inhibit T-cell responses by cell-contact dependent mechanisms (36) and by metabolic disruption (33, 39, 41).

Results from pre-clinical murine and humanized models convinced investigators that Tregs can be used to control graft-versus-host disease (GvHD) as well as organ rejection, or to treat autoimmune diseases (47, 48). Good-manufacturing-practice (GMP)-grade protocols to isolate and expand human Tregs *in vitro* without losing their suppressive function and to generate human Ag-specific Tregs have been established allowing translation of Treg-based therapy to the clinical practice.

COMPLETED AND ONGOING Treg-BASED CLINICAL TRIALS

Treg-based therapy has been used for the first time to prevent GvHD in patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT). Six independent trials, using either FOXP3$^+$ Tregs or Tr1 cells, have been concluded, and all of them showed the feasibility and safety of Treg-based approaches (49–54) (**Table 1**). In five of these trials, either freshly isolated (51, 54, 55) or *ex vivo* expanded FOXP3$^+$ Tregs (49, 50) were infused in patients undergoing allo-HSCT for onco-hematological diseases. Three of these trials also indicated the potential efficacy of the treatment. Brunstein et al. (50) reported a decreased incidence of grade II–IV GvHD as compared to historical controls when umbilical cord blood (UBC)-derived Tregs were injected, without increased risk of infections. Similarly, Di Ianni et al. (51) described few cases of low grade GvHD (2 out of 26 patients) and no development of chronic GvHD in patients injected with un-manipulated peripheral Tregs. More recently, it has been reported that in Treg-treated patients, the cumulative incidence of relapse was significantly lower than in historical controls (54). Previous trials based on the adoptive transfer of alloAgs-specific anergic T cells generated *in vitro* in the presence of Belatacept (CTLA-4-Ig) to prevent GvHD after allo-HSCT were performed (56, 57). Later, it was demonstrated that alloAgs-specific anergic T cells generated with CTLA-4-Ig contained a small fraction of FOXP3$^+$ Tregs (58).

Our group has completed a phase-I clinical trial in which IL-10-anergized T cells (IL-10 DLI) containing Tr1 cells were injected in patients undergoing haploidentical-HSCT (53). Donor-derived IL-10-anergized T cells specific for host allo-Ags were generated *in vitro* through activation of T cells by host-derived APCs in the presence of exogenous IL-10 (60). An improved protocol for the generation of Tr1 cells, which foresees the use of tolerogenic dendritic cells (DC-10)(61), has been developed (60, 62). Although a small cohort of patients was treated, our results demonstrated that after infusion of IL-10 DLI no acute adverse events and only mild GvHD (grade II or III responsive to therapy) were observed. Furthermore, the treatment accelerated immune reconstitution after transplant and long-lasting disease remission (53).

The above-mentioned trials paved the way to a wider application of Tregs as advanced medical products for the treatment of autoimmunity in type 1 diabetes (T1D), inflammatory diseases, and rejection after solid organ transplantation. *Ex vivo* expanded CD4$^+$CD25^{hi}CD127$^-$ Tregs were administered to children with recent onset T1D in a phase-I trial (59) (**Table 1**). The procedure appeared to be safe, as no adverse reactions related to the treatment were reported. However, the few data available do not allow drawing conclusions on the clinical relevance of the procedure (59). The group of Bluestone is currently testing the safety of *ex vivo* expanded polyclonal CD4$^+$CD25^{hi}CD127$^{low/-}$ Tregs in a phase-I clinical trial (NCT01210664) in which increasing doses of Tregs will be injected in recent onset adult T1D patients (63). A phase-I/IIa clinical study in which Ag-specific Tr1 cell clones were used to treat patients with Crohn's Disease has been recently reported. Overall, a response was observed in 40% of patients, with stronger effect in the group of patients who received the lowest Tr1 cell dose (40) (**Table 1**). The France-based company TxCell is currently heading a consortium dedicated to the clinical development of collagen-specific Tr1 cells (Col-Treg) to be tested in a first-in-man clinical study for severe and refractory autoimmune uveitis scheduled to start in 2016.[1]

The power of Tregs in inducing tolerance to allo-Ags after solid organ transplantation is currently under evaluation. In liver transplantation, several clinical trials are ongoing using polyclonal expanded Tregs with or without rapamycin (Treg trial, NCT01624077, ThRIL trial NCT02166177) or donor-specific expanded Tregs (darTreg: deLTA Trial NCT02188719, and ARTEMIS Trial NCT02474199). In addition, *ex vivo* expanded autologous polyclonal CD4$^+$CD25$^+$ Tregs are currently tested in the context of kidney transplantation (TRACT Trial, NCT 02145325 and TASK Trial NCT0288931). Moreover, an ambitious project in which the efficacy of different immune-regulatory cells, including polyclonal expanded Tregs with or without rapamycin (One Treg1 Trial, NCT02129881, ONE nTreg13 Trial NCT02371434), darTreg cells (DART Trial NCT02244801), and donor-specific T cells anergized in the presence of Belatacept (NCT02091232), and Tr1 cells induced with DC-10, will be compared in kidney transplant recipients ("The ONE study," discussed in details below) is currently ongoing. Results of these trials will definitely address the safety of this approach and will also provide hints on their efficacy as therapeutic agents.

OPEN ISSUES IN Treg-BASED IMMUNOTHERAPY

Despite the promising results obtained from the above-mentioned pilot clinical trials, many open questions remain on the best

[1] http://www.txcell.com

TABLE 1 | Completed Treg-based clinical trials.

Trial ID	Cell product	Disease	Safety	Efficacy	Reference
N.A.	*In vitro* expanded donor-derived $CD4^+CD25^{high}CD127^-$ Tregs	GvHD after HLA-matched sibling HSCT for hematological malignancies	Yes	N.A.	(49)
N.A.	Freshly isolated donor-derived $CD4^+CD25^{high}CD127^-$ Tregs	GvHD after allo-HSCT for hematological malignancies	Yes	N.A.	(55)
NCT00602693	*In vitro* expanded UCB-derived $CD4^+CD25^+$ Tregs	GvHD after DUCBT for hematological malignancies	Yes	Yes	(50)
CEAS Umbria Protocol No 01/08 2008	Freshly isolated donor-derived $CD4^+CD25^+$ Tregs	GvHD after haplo-HSCT for hematological malignancies	Yes	Yes	(51)
CEAS Umbria Protocol No 0108	Freshly isolated donor-derived $CD4^+CD25^+$ Tregs	GvHD after haplo-HSCT for hematological malignancies	Yes	Yes	(54)
NKEBN/8/2010	*In vitro* expanded autologous $CD4^+CD25^{high}CD127^-$ Tregs	Pediatric recent onset T1D	Yes	N.A.	(59)
ALT-TEN	IL-10 DLI donor-derived IL-10 anergized T cells	GvHD after haplo-HSCT for hematological malignancies	yes	N.A.	(53)
CATS 1	Autologous OVA-specific Tr1 cell clones; Ovasave®	Refractory Crohn's Disease	Yes	Yes	(40)

GvHD, graft-versus-host disease; haplo-HSCT, haploidentical-hematopoietic stem cell transplantation; UCB, umbilical cord blood; DUCBT, double umbilical cord blood transplant; allo-HSCT, allogeneic-HSCT; T1D, type 1 diabetes; DLI, donor lymphocyte infusion; OVA, ovalbumin.

source and subtype of Tregs to be administered, the survival of these cells in the host, and their mechanisms of action.

The ONE Study[2] is a large-scale, collaborative project funded by the Seventh Framework Programme (FP7) of the European Commission, envisioned to ascertain which immuno-modulatory cell type (among *ex vivo* isolated and *in vitro* expanded polyclonal or allo-specific $FOXP3^+$ Tregs, Tr1 cells, and tolerogenic APCs) is best fit to induce tolerance to allo-Ags in patients receiving kidney transplants (64, 65). Results from this study will define which regulatory cell population is the most efficient in promoting graft acceptance and tolerance.

Recent work has led to the identification of specialized subsets of Tregs, which reside in peripheral tissues, including skin, intestinal mucosa, adipose tissue, autoimmune target tissues, and injured muscle (66). Although tissue-resident Tregs represent a small fraction of total Tregs, their peculiar phenotype and function confer the ability to regulate tissue-specific physiological and pathological processes. Therapies aimed at targeting tissue-specific Tregs may potentially allow the local control of the disease, without affecting systemic immunity. Although the clinical application of tissue-resident Tregs remains unexplored, the possibility of exploiting these subsets deserves to be investigated in the near future.

One pre-requisite for Treg-based therapies is their *in vivo* viability and persistence. In a clinical trial in allo-HSCT, upon *in vivo* infusion Tregs were no longer detected in the circulation after 2 weeks (50). Similarly, in T1D patients, *in vitro* expanded $CD4^+CD25^+CD127^-$ Tregs labeled with deuterium were found at high frequency in the peripheral blood 2 weeks after injection, then declined but they were still detectable at low frequency 6 months after therapy [Bluestone JA, unpublished data presented at FOCIS Annual Meeting 2015]. It is still unclear whether infused Tregs migrate to tissues or have limited *in vivo* survival because of *in vitro* expansion. In IL-10 DLI-treated patients, we found an expansion of circulating granzyme B/IL-10 and CD49b/LAG-3-expressing $CD4^+$ T cells that progressively increased during follow up. The percentages of these cells were higher in the IL-10 DLI-treated long-term surviving patients (up to 8 years after haplo-HSCT), as compared to those in healthy subjects (53). These data support the hypothesis that IL-10 DLI infusion supports either Tr1 cell expansion, or the *de novo* induction of Tr1 cells.

Increasing evidence suggests that FOXP3-expressing Tregs are intrinsically plastic (67–69). Therefore, the risk of their *in vivo* conversion into Teff cells under inflammatory conditions, and consequent loss of their suppressive ability, cannot be ignored. To allow safe clinical application of Tregs, investigators are currently trying to address this issue. For example, rapamycin permits the *in vitro* expansion of $FOXP3^+$ Tregs, while impairing the proliferation of contaminating Teff cells (70, 71). Importantly, rapamycin-expanded $FOXP3^+$ Tregs maintain their regulatory phenotype, even upon exposure to a pro-inflammatory environment (72, 73). Clinical-grade Treg expansion protocols with rapamycin have been implemented for ongoing clinical trials under the umbrella of the European consortium "The ONE study" (65, 74). On the same line, in order to avoid infusion of Teff cell contaminants potentially allo-reactive, allo-anergization of T cells in the presence of costimulatory blockade with Belatacept has been proposed (58) and is currently being tested (NCT02091232).

One major concern for the use of immunotherapy with Tregs to control GvHD after allo-HSCT for hematological malignancies is the potential inhibition of the beneficial graft-versus-leukemia (GvL) effects. Results from one of the completed phase II trials showed that in $CD25^+$ Treg-treated patients the cumulative incidence of relapse was significantly lower than in historical controls. The Authors proposed that the failure of human $CD4^+CD25^+$ Tregs

[2]www.onestudy.org

to home to the bone marrow does not hamper the GvL activity of the donor conventional T cells (54). Although promising, these results are still preliminary and required further confirmation.

UP-COMING CHALLENGES IN Treg-BASED IMMUNOTHERAPY

As previously mentioned, increasing evidence suggests that FOXP3$^+$ Tregs are a heterogeneous population, including several specialized subtypes, making it difficult to choose the "right" variety of cells for specific treatments. To overcome this limitation, we developed a novel and efficient method to generate homogeneous populations of human FOXP3-expressing Tregs by Lentiviral-Vector (LV)-mediated *hFOXP3* gene transfer into conventional CD4$^+$ T cells, hereafter indicated as CD4^{FOXP3} T cells. Constitutive over-expression of FOXP3 generates functional and stable FOXP3$^+$ Treg-like cells, with potent *in vitro* and *in vivo* suppressive activity, reduced proliferative capacity and cytokine production (75, 76). CD4^{FOXP3} T cells generated from naïve CD4$^+$ T cells have stable expression of FOXP3 in steady state and inflammatory conditions, whereas CD4^{FOXP3} T cells generated from memory cells show reduced percentage of FOXP3$^+$ T cells upon activation, especially in the presence of inflammatory cytokines. The instability of FOXP3 expression in memory CD4^{FOXP3} T cells results in weaker suppressive function and increased proliferative capacity, confirming that acquisition of Treg functions is dependent on stable FOXP3 expression (76).

Despite recent advances in the establishment of protocols to efficiently generate Allo Ag-specific Tr1 cells *in vitro*, the resulting populations still contain contaminants that could potentially limit the *in vivo* efficacy of Tr1 cells (60, 61). The recent discovery of CD49b and LAG-3 as specific biomarkers of Tr1 cells that allow the isolation of Tr1 cells from *in vitro* Tr1-polarized populations (32) will open the possibility to select human Tr1 cells from mixed cultures. As an alternative to obtain a large and homogeneous population of Tr1 cells, the LV-mediated *hIL-10* gene transfer has been used to convert conventional T cells into Tr1-like cells, termed CD4$^{IL\text{-}10}$ (77). CD4$^{IL\text{-}10}$ cells mirror the phenotype and function of Tr1 cells and suppress xeno-GvHD (77). These findings pave the way for adoptive cell therapy with FOXP3- or IL-10-engineered T cells in patients with autoimmune disorders and in patients undergoing allogeneic organ or HSC transplantation. Issues related to undesired effects of therapy with genetically modified cells, such as induction of general immunosuppression, impairment of immune reconstitution, and GvL activity in the context of allo-HSCT for hematological diseases are still under investigation.

In humanized pre-clinical models, allo-specific Tregs are more effective in preventing graft rejection as compared to polyclonal Tregs (78, 79). It is possible to select allo-specific Tregs from peripheral blood according to the expression of early activation markers and/or then *in vitro* expand them (78, 80). Moreover, a GMP-grade protocol to selectively expand human allo-specific Tregs using CD40L-activated B cells has also been established (79). As an alternative, ectopic expression of genes encoding for TCR with known specificity has been proposed. Forced expression of specific TCRs confers the desired specificity to human polyclonal Tregs. As a proof-of-concept, it has been shown that TCR or chimeric receptor specific for tumor Ags can be introduced in human polyclonal Tregs, conferring them the ability to potently suppress anti-tumor responses (81–83). It was also proposed to generate Ag-specific Tregs starting from conventional T cells engineered to over-express both *hFOXP3* and TCR specific for a birch pollen allergen-derived peptide Betv1. The resulting T cells acquired a Treg phenotype and suppressed T-cell responses in an Ag-specific manner (84). Despite these data provided the proof-of-concept for such approaches, several questions regarding the potential clinical application of these engeneered T cells have to be addressed. Among others, one of the major concerns regards the need to eliminate endogenous TCRs to avoid double specificity and the risk of bystander undesired suppressive function. An interesting and promising approach to overcome this limitation is LV-mediated gene transfer of either *hFOXP3* or *hIL-10* in Ag-experienced T cells isolated from peripheral blood.

An additional crucial question for the success of Treg-based therapy, in particular in the context of solid organ transplantation, is how immunosuppressive treatments affect Treg survival and function. The impact of current immunosuppressive drugs on Tregs has been extensively reviewed in Ref. (48, 85). The general consensus is that calcineurin inhibitors are likely to be detrimental to Tregs, whereas drugs such as rapamycin or mycophenolate mofetil (MMF) preserve Tregs *in vivo*. However, indications will come from the results of "The ONE study" in which Tregs will be infused in patients receiving kidney transplantation and standard triple-therapy protocol (prednisolone, MMF, and tacrolimus) (65).

Finally, the heterogeneity of the parameters selected to monitor Treg activity in the recently completed trials hampers comparison of the results. To overcome this limitation, the EU COST Action "BM1305: action to focus and accelerate cell-based tolerance-inducing therapies[3]" has been funded to identify shared and disease specific biomarkers of tolerance in patients undergoing Treg-based therapies. This action is complementary to "The ONE study" and aims at defining general tolerance signatures and standardized immune monitoring protocols (65).

CONCLUDING REMARKS

The discovery that Tregs modulate immune responses led to the idea that they could be developed as a therapeutic tool to promote/restore tolerance to transplanted grafts and in inflammatory and autoimmune diseases. The recent clinical trials proved the safety of this approach and suggested a possible therapeutic effect. Thus far, the major challenges in the field were to expand hard-to-grow polyclonal Tregs to great purity, and to generate Ag-specific Tregs. Despite technical advances in the field, many questions relating to Treg-based therapies remain unanswered: Which cell type to be used? Which schedule of cell infusion? How long Tregs will survive *in vivo*? How long their effect will last? What is their mechanism of action? Do they interfere with GvL in the context of allo-HSCT? Moreover, reliable biomarkers of tolerance and

[3] www.afactt.eu

standardized methods to evaluate the efficacy of Treg-based therapy are required to compare the outcome of present and future trials. To address these questions, close collaboration between groups in the field is required to allow the systematic comparison of Tregs and outcomes of cell therapy trials.

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REFERENCES

1. Bluestone JA, Trotta E, Xu D. The therapeutic potential of regulatory T cells for the treatment of autoimmune disease. *Expert Opin Ther Targets* (2015) **19**(8):1091–103. doi:10.1517/14728222.2015.1037282
2. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* (1995) **155**:1151–64.
3. Groux H, O'garra A, Bigler M, Rouleau M, Antonenko S, De Vries JE, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* (1997) **389**:737–42. doi:10.1038/39614
4. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* (2010) **10**:490–500. doi:10.1038/nri2785
5. Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, et al. Regulatory T cells: recommendations to simplify the nomenclature. *Nat Immunol* (2013) **14**:307–8. doi:10.1038/ni.2554
6. Schmetterer KG, Neunkirchner A, Pickl WF. Naturally occurring regulatory T cells: markers, mechanisms, and manipulation. *FASEB J* (2012) **26**:2253–76. doi:10.1096/fj.11-193672
7. Hill JA, Feuerer M, Tash K, Haxhinasto S, Perez J, Melamed R, et al. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* (2007) **27**:786–800. doi:10.1016/j.immuni.2007.09.010
8. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* (2010) **184**:3433–41. doi:10.4049/jimmunol.0904028
9. Akimova T, Beier UH, Wang L, Levine MH, Hancock WW. Helios expression is a marker of T cell activation and proliferation. *PLoS One* (2011) **6**:e24226. doi:10.1371/journal.pone.0024226
10. Gottschalk RA, Corse E, Allison JP. Expression of Helios in peripherally induced Foxp3+ regulatory T cells. *J Immunol* (2012) **188**:976–80. doi:10.4049/jimmunol.1102964
11. Wieczorek G, Asemissen A, Model F, Turbachova I, Floess S, Liebenberg V, et al. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. *Cancer Res* (2009) **69**:599–608. doi:10.1158/0008-5472.CAN-08-2361
12. Ohkura N, Hamaguchi M, Morikawa H, Sugimura K, Tanaka A, Ito Y, et al. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity* (2012) **37**:785–99. doi:10.1016/j.immuni.2012.09.010
13. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* (2000) **192**:303–10. doi:10.1084/jem.192.2.303
14. McHugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, et al. CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* (2002) **16**:311–23. doi:10.1016/S1074-7613(02)00280-7
15. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* (2007) **110**:1225–32. doi:10.1182/blood-2006-12-064527
16. Kubach J, Lutter P, Bopp T, Stoll S, Becker C, Huter E, et al. Human CD4+CD25+ regulatory T cells: proteome analysis identifies galectin-10 as a novel marker essential for their anergy and suppressive function. *Blood* (2007) **110**:1550–8. doi:10.1182/blood-2007-01-069229
17. Tran DQ, Andersson J, Hardwick D, Bebris L, Illei GG, Shevach EM. Selective expression of latency-associated peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated human FOXP3+ regulatory T cells allows for their purification from expansion cultures. *Blood* (2009) **113**:5125–33. doi:10.1182/blood-2009-01-199950
18. Tran DQ, Andersson J, Wang R, Ramsey H, Unutmaz D, Shevach EM. GARP (LRRC32) is essential for the surface expression of latent TGF-beta on platelets and activated FOXP3+ regulatory T cells. *Proc Natl Acad Sci U S A* (2009) **106**:13445–50. doi:10.1073/pnas.0901944106
19. Ito T, Hanabuchi S, Wang YH, Park WR, Arima K, Bover L, et al. Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. *Immunity* (2008) **28**:870–80. doi:10.1016/j.immuni.2008.03.018
20. Nakamura K, Kitani A, Fuss I, Pedersen A, Harada N, Nawata H, et al. TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. *J Immunol* (2004) **172**:834–42. doi:10.4049/jimmunol.172.2.834
21. Stockis J, Colau D, Coulie PG, Lucas S. Membrane protein GARP is a receptor for latent TGF-beta on the surface of activated human Treg. *Eur J Immunol* (2009) **39**:3315–22. doi:10.1002/eji.200939684
22. Chaturvedi V, Collison LW, Guy CS, Workman CJ, Vignali DA. Cutting edge: human regulatory T cells require IL-35 to mediate suppression and infectious tolerance. *J Immunol* (2011) **186**:6661–6. doi:10.4049/jimmunol.1100315
23. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* (2004) **21**:589–601. doi:10.1016/j.immuni.2004.09.002
24. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* (2008) **322**:271–5. doi:10.1126/science.1160062
25. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol* (2007) **8**:1353–62. doi:10.1038/ni1536
26. Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* (2007) **204**:1257–65. doi:10.1084/jem.20062512
27. Bopp T, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, et al. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* (2007) **204**:1303–10. doi:10.1084/jem.20062129
28. Baecher-Allan C, Wolf E, Hafler DA. MHC class II expression identifies functionally distinct human regulatory T cells. *J Immunol* (2006) **176**:4622–31. doi:10.4049/jimmunol.176.8.4622
29. Beriou G, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C, et al. IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood* (2009) **113**:4240–9. doi:10.1182/blood-2008-10-183251
30. Hoffmann P, Eder R, Boeld TJ, Doser K, Piseshka B, Andreesen R, et al. Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to

homogeneous regulatory T-cell lines upon in vitro expansion. *Blood* (2006) **108**:4260–7. doi:10.1182/blood-2006-06-027409
31. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* (2009) **30**:899–911. doi:10.1016/j.immuni.2009.03.019
32. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med* (2013) **19**:739–46. doi:10.1038/nm.3179
33. Gregori S, Goudy KS, Roncarolo MG. The cellular and molecular mechanisms of immuno-suppression by human type 1 regulatory T cells. *Front Immunol* (2012) **3**:30. doi:10.3389/fimmu.2012.00030
34. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M. Tr1 cells and the counter-regulation of immunity: natural mechanisms and therapeutic applications. *Curr Top Microbiol Immunol* (2014) **380**:39–68. doi:10.1007/978-3-662-43492-5_3
35. Bacchetta R, Sartirana C, Levings MK, Bordignon C, Narula S, Roncarolo MG. Growth and expansion of human T regulatory type 1 cells are independent from TCR activation but require exogenous cytokines. *Eur J Immunol* (2002) **32**:2237–45. doi:10.1002/1521-4141(200208)32:8<2237::AID-IMMU2237>3.0.CO;2-2
36. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* (2004) **199**:1567–75. doi:10.1084/jem.20032058
37. Haringer B, Lozza L, Steckel B, Geginat J. Identification and characterization of IL-10/IFN-gamma-producing effector-like T cells with regulatory function in human blood. *J Exp Med* (2009) **206**:1009–17. doi:10.1084/jem.20082238
38. Bergmann C, Strauss L, Zeidler R, Lang S, Whiteside TL. Expansion and characteristics of human T regulatory type 1 cells in co-cultures simulating tumor microenvironment. *Cancer Immunol Immunother* (2007) **56**:1429–42. doi:10.1007/s00262-007-0280-9
39. Mandapathil M, Szczepanski MJ, Szajnik M, Ren J, Jackson EK, Johnson JT, et al. Adenosine and prostaglandin E2 cooperate in the suppression of immune responses mediated by adaptive regulatory T cells. *J Biol Chem* (2010) **285**:27571–80. doi:10.1074/jbc.M110.127100
40. Desreumaux P, Foussat A, Allez M, Beaugerie L, Hebuterne X, Bouhnik Y, et al. Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn' disease. *Gastroenterology* (2012) **143**(1207–1217):e1201–2. doi:10.1053/j.gastro.2012.07.116
41. Mascanfroni ID, Takenaka MC, Yeste A, Patel B, Wu Y, Kenison JE, et al. Metabolic control of type 1 regulatory T cell differentiation by AHR and HIF1-alpha. *Nat Med* (2015) **21**:638–46. doi:10.1038/nm.3868
42. Vieira PL, Christensen JR, Minaee S, O'neill EJ, Barrat FJ, Boonstra A, et al. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *J Immunol* (2004) **172**:5986–93. doi:10.4049/jimmunol.172.10.5986
43. Levings MK, Gregori S, Tresoldi E, Cazzaniga S, Bonini C, Roncarolo MG. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. *Blood* (2005) **105**:1162–9. doi:10.1182/blood-2004-03-1211
44. Brun V, Bastian H, Neveu V, Foussat A. Clinical grade production of IL-10 producing regulatory Tr1 lymphocytes for cell therapy of chronic inflammatory diseases. *Int Immunopharmacol* (2009) **9**:609–13. doi:10.1016/j.intimp.2009.01.032
45. Brun V, Neveu V, Pers YM, Fabre S, Quatannens B, Bastian H, et al. Isolation of functional autologous collagen-II specific IL-10 producing Tr1 cell clones from rheumatoid arthritis blood. *Int Immunopharmacol* (2011) **11**:1074–8. doi:10.1016/j.intimp.2011.03.001
46. Magnani CF, Alberigo G, Bacchetta R, Serafini G, Andreani M, Roncarolo MG, et al. Killing of myeloid APCs via HLA class I, CD2 and CD226 defines a novel mechanism of suppression by human Tr1 cells. *Eur J Immunol* (2011) **41**:1652–62. doi:10.1002/eji.201041120
47. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat Rev Immunol* (2007) **7**:585–98. doi:10.1038/nri2138
48. Tang Q, Bluestone JA, Kang SM. CD4(+)Foxp3(+) regulatory T cell therapy in transplantation. *J Mol Cell Biol* (2012) **4**:11–21. doi:10.1093/jmcb/mjr047
49. Trzonkowski P, Bieniaszewska M, Juscinska J, Dobyszuk A, Krzystyniak A, Marek N, et al. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. *Clin Immunol* (2009) **133**:22–6. doi:10.1016/j.clim.2009.06.001
50. Brunstein CG, Miller JS, Cao Q, Mckenna DH, Hippen KL, Curtsinger J, et al. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* (2011) **117**(3):1061–70. doi:10.1182/blood-2010-07-293795
51. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood* (2011) **117**:3921–8. doi:10.1182/blood-2010-10-311894
52. Edinger M, Hoffmann P. Regulatory T cells in stem cell transplantation: strategies and first clinical experiences. *Curr Opin Immunol* (2011) **23**:679–84. doi:10.1016/j.coi.2011.06.006
53. Bacchetta R, Lucarelli B, Sartirana C, Gregori S, Lupo Stanghellini MT, Miqueu P, et al. Immunological outcome in haploidentical-HSC transplanted patients treated with IL-10-anergized donor T cells. *Front Immunol* (2014) **5**:16. doi:10.3389/fimmu.2014.00016
54. Martelli MF, Di Ianni M, Ruggeri L, Falzetti F, Carotti A, Terenzi A, et al. HLA-haploidentical transplantation with regulatory and conventional T cell adoptive immunotherapy prevents acute leukemia relapse. *Blood* (2014) **124**(4):638–44. doi:10.1182/blood-2014-03-564401
55. Edinger M. Treg cells in allogeneic stem cell transplantation. Keystone Symposia on Molecular and Cellular Biology Regulatory T cells (C5) 2009 Abstract 028 (2009).
56. Guinan EC, Boussiotis VA, Neuberg D, Brennan LL, Hirano N, Nadler LM, et al. Transplantation of anergic histoincompatible bone marrow allografts. *N Engl J Med* (1999) **340**:1704–14. doi:10.1056/NEJM199906033402202
57. Davies JK, Gribben JG, Brennan LL, Yuk D, Nadler LM, Guinan EC. Outcome of alloanergized haploidentical bone marrow transplantation after ex vivo costimulatory blockade: results of 2 phase 1 studies. *Blood* (2008) **112**:2232–41. doi:10.1182/blood-2008-03-143636
58. Davies JK, Nadler LM, Guinan EC. Expansion of allospecific regulatory T cells after anergized, mismatched bone marrow transplantation. *Sci Transl Med* (2009) **1**(1):1ra3. doi:10.1126/scitranslmed.3000153
59. Marek-Trzonkowska N, Mysliwiec M, Dobyszuk A, Grabowska M, Techmanska I, Juscinska J, et al. Administration of CD4+CD25highCD127-regulatory T cells preserves beta-cell function in type 1 diabetes in children. *Diabetes Care* (2012) **35**:1817–20. doi:10.2337/dc12-0038
60. Bacchetta R, Gregori S, Serafini G, Sartirana C, Schulz U, Zino E, et al. Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy. *Haematologica* (2010) **95**:2134–43. doi:10.3324/haematol.2010.025825
61. Gregori S, Tomasoni D, Pacciani V, Scirpoli M, Battaglia M, Magnani CF, et al. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* (2010) **116**:935–44. doi:10.1182/blood-2009-07-234872
62. Petrelli A, Et BS, Mfarrej BG, Paganelli A, Spotti D, Caldara R, et al. Generation of donor-specific T regulatory type 1 cells from patients on dialysis for cell therapy after kidney transplantation. *Transplantation* (2015) **99**(8):1582–9. doi:10.1097/TP.0000000000000751
63. Putnam A, Lares A, Lee M, Liu W, Herold KC, Gitelman S, et al. Results from 10 study participant treated with ex vivo expanded CD4+CD127low/-CD25+ polyclonal Tregs in a phase I clinical trial for the treatment of recent-onset type 1 diabetes. *Clin Immunol* (2013) **104**:156.
64. Leslie M. Immunology. Regulatory T cell get their chance to shine. *Science* (2011) **332**:1020–1. doi:10.1126/science.332.6033.1020
65. Hutchinson JA, Geissler EK. Now or never? The case for cell-based immunosuppression in kidney transplantation. *Kidney Int* (2015) **87**:1116–24. doi:10.1038/ki.2015.50
66. Lu J, Meng H, Zhang A, Yang J, Zhang X. Phenotype and function of tissue-resident unconventional Foxp3-expressing CD4(+) regulatory T cells. *Cell Immunol* (2015) **297**:53–9. doi:10.1016/j.cellimm.2015.06.005
67. Zhou X, Bailey-Bucktrout S, Jeker LT, Bluestone JA. Plasticity of CD4(+) FoxP3(+) T cells. *Curr Opin Immunol* (2009) **21**:281–5. doi:10.1016/j.coi.2009.05.007

68. Da Silva Martins M, Piccirillo CA. Functional stability of Foxp3+ regulatory T cells. *Trends Mol Med* (2012) **18**:454–62. doi:10.1016/j.molmed.2012.06.001
69. MacDonald KG, Orban PC, Levings MK. T regulatory cell therapy in transplantation: stability, localization and functional specialization. *Curr Opin Organ Transplant* (2012) **17**:343–8. doi:10.1097/MOT.0b013e328355aaaf
70. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* (2005) **105**:4743–8. doi:10.1182/blood-2004-10-3932
71. Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaupper T, Roncarolo MG. Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol* (2006) **177**:8338–47. doi:10.4049/jimmunol.177.12.8338
72. Tresoldi E, Dell'albani I, Stabilini A, Jofra T, Valle A, Gagliani N, et al. Stability of human rapamycin-expanded CD4(+)CD25(+) T regulatory cells. *Haematologica* (2011) **96**:1357–65. doi:10.3324/haematol.2011.041483
73. Scotta C, Esposito M, Fazekasova H, Fanelli G, Edozie FC, Ali N, et al. Differential effects of rapamycin and retinoic acid on expansion, stability and suppressive qualities of human CD4(+)CD25(+)FOXP3(+) T regulatory cell subpopulations. *Haematologica* (2013) **98**:1291–9. doi:10.3324/haematol.2012.074088
74. Van Der Net JB, Bushell A, Wood KJ, Harden PN. Regulatory T cells: first steps of clinical application in solid organ transplantation. *Transpl Int* (2015). doi:10.1111/tri.12608
75. Allan SE, Alstad AN, Merindol N, Crellin NK, Amendola M, Bacchetta R, et al. Generation of potent and stable human CD4+ T regulatory cells by activation-independent expression of FOXP3. *Mol Ther* (2008) **16**:194–202. doi:10.1038/sj.mt.6300341
76. Passerini L, Mel ER, Sartirana C, Fousteri G, Bondanza A, Naldini L, et al. CD4+ T cells from IPEX patients convert into functional and stable regulatory T cells by FOXP3 gene transfer. *Sci Transl Med* (2013) **5**(215):215ra174. doi:10.1126/scitranslmed.3007320
77. Andolfi G, Fousteri G, Rossetti M, Magnani CF, Jofra T, Locafaro G, et al. Enforced IL-10 expression confers type 1 regulatory T cell (Tr1) phenotype and function to human CD4(+) T cells. *Mol Ther* (2012) **20**:1778–90. doi:10.1038/mt.2012.71
78. Sagoo P, Ali N, Garg G, Nestle FO, Lechler RI, Lombardi G. Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells. *Sci Transl Med* (2011) **3**(83):83ra42. doi:10.1126/scitranslmed.3002076
79. Putnam AL, Safinia N, Medvec A, Laszkowska M, Wray M, Mintz MA, et al. Clinical grade manufacturing of human alloantigen-reactive regulatory T cells for use in transplantation. *Am J Transplant* (2013) **13**:3010–20. doi:10.1111/ajt.12433
80. Noyan F, Lee YS, Zimmermann K, Hardtke-Wolenski M, Taubert R, Warnecke G, et al. Isolation of human antigen-specific regulatory T cells with high suppressive function. *Eur J Immunol* (2014) **44**:2592–602. doi:10.1002/eji.201344381
81. Hombach AA, Kofler D, Rappl G, Abken H. Redirecting human CD4+CD25+ regulatory T cells from the peripheral blood with pre-defined target specificity. *Gene Ther* (2009) **16**:1088–96. doi:10.1038/gt.2009.75
82. Brusko TM, Koya RC, Zhu S, Lee MR, Putnam AL, Mcclymont SA, et al. Human antigen-specific regulatory T cells generated by T cell receptor gene transfer. *PLoS One* (2010) **5**:e11726. doi:10.1371/journal.pone.0011726
83. Wan Q, Kozhaya L, Imberg K, Mercer F, Zhong S, Krogsgaard M, et al. Probing the effector and suppressive functions of human T cell subsets using antigen-specific engineered T cell receptors. *PLoS One* (2013) **8**:e56302. doi:10.1371/journal.pone.0056302
84. Schmetterer KG, Haiderer D, Leb-Reichl VM, Neunkirchner A, Jahn-Schmid B, Kung HJ, et al. Bet v 1-specific T-cell receptor/forkhead box protein 3 transgenic T cells suppress Bet v 1-specific T-cell effector function in an activation-dependent manner. *J Allergy Clin Immunol* (2011) **127**:.e231–3. doi:10.1016/j.jaci.2010.10.023
85. Battaglia M. Potential T regulatory cell therapy in transplantation: how far have we come and how far can we go? *Transpl Int* (2010) **23**:761–70. doi:10.1111/j.1432-2277.2010.01127.x

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MINI REVIEW
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Opposing roles of interferon-gamma on cells of the central nervous system in autoimmune neuroinflammation

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Multiple sclerosis (MS) is the principal cause of autoimmune neuroinflammation in humans, and its animal model, experimental autoimmune encephalomyelitis (EAE), is widely used to gain insight about their immunopathological mechanisms for and the development of novel therapies for MS. Most studies on the role of interferon (IFN)-γ in the pathogenesis and progression of EAE have focused on peripheral immune cells, while its action on central nervous system (CNS)-resident cells has been less explored. In addition to the well-known proinflammatory and damaging effects of IFN-γ in the CNS, evidence has also endowed this cytokine both a protective and regulatory role in autoimmune neuroinflammation. Recent investigations performed in this research field have exposed the complex role of IFN-γ in the CNS uncovering unexpected mechanisms of action that underlie these opposing activities on different CNS-resident cell types. The mechanisms behind these two-faced effects of IFN-γ depend on dose, disease phase, and cell development stage. Here, we will review and discuss the dual role of IFN-γ on CNS-resident cells in EAE highlighting its protective functions and the mechanisms proposed.

Keywords: interferon-gamma, experimental autoimmune encephalomyelitis, multiple sclerosis, glial cells, neurons, central nervous system, neuroinflammation

INTRODUCTION

Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), are chronic autoimmune diseases of the central nervous system (CNS) characterized by inflammatory infiltrates, demyelination and neurological damage (1, 2). MS and EAE were initially considered to be mediated by interferon (IFN)-γ-expressing T helper (Th) 1 cells (3, 4). Currently, it is most widely accepted that several innate and adaptive immune cell types and immunomodulatory molecules contribute to the disease development and progression (5). Moreover, several studies have challenged the notion that IFN-γ is only pathogenic, and accumulative evidence attributes it a protective role in EAE and MS (6–8). In this same journal research topic, we have reviewed recent data supporting a stage-specific participation of IFN-γ in MS and EAE providing a plausible explanation for previous conflicting results and a model whereby this cytokine can both promote and limit the development of

these pathologies (8). However, the majority of these studies have focused on the roles of IFN-γ in immune cells, while its activity in CNS-resident cells remains less explored. In this review, we will begin by discussing evidence that reports opposite roles of IFN-γ in the CNS during EAE development. Then, we will review both the inflammatory and protective effects of IFN-γ on glial cells and neurons in EAE.

OPPOSING EFFECTS OF IFN-γ IN THE CNS DURING EAE

Early studies demonstrated that IFN-γ can activate CNS-resident cells and induce expression of major histocompatibility complex (MHC) molecules (3, 9). Direct injection of IFN-γ into the rat CNS induced inflammation and cellular infiltration similar to that observed in EAE (10–13) and potentiated the demyelination process (13). Furthermore, demyelination occurred in transgenic mice expressing IFN-γ under the control of the myelin basic protein (MBP) promoter (14–16). On the contrary, animals with EAE that were injected with IFN-γ systemically or directly into the CNS showed amelioration of clinical symptoms (17–20). Therefore, despite early data reporting an inflammatory effect of IFN-γ in the CNS during EAE development, accumulating evidence has also demonstrated a neuroprotective activity for IFN-γ in this disease.

Classical EAE is characterized by an ascending progressive paralysis dominated by inflammatory lesions in the white matter of the spinal cord and limited brain inflammation (2, 21). However, the absence of either IFN-γ or its receptor (IFNGR) leads to the development of atypical EAE symptoms such as head tilting, ataxia, dystonia, spasticity, and axial-rotation suggestive of brain-associated damage (19, 22–24). This atypical EAE is associated with greater encephalitogenicity of Th1 and Th17 cells and enhanced demyelination in the brainstem and cerebellum (19, 24). Even more, some studies have shown that IFN-γ may exert opposite effects in the brain and spinal cord determining the regional localization of lesions and inflammation in EAE. According to this evidence, IFN-γ induces inflammation in the spinal cord but protection in the brain (21, 25, 26). The mechanisms underlying these differential effects of IFN-γ may involve the regulation of the expression of specific chemokines in the brain versus spinal cord that restrain encephalitogenic T cell brain infiltration (19, 24, 25).

IFN-γ may also exert differential effects on the blood–brain barrier (BBB) and blood-spinal cord barrier (BSCB), resulting in opposite effects on their function and integrity. Supporting this hypothesis, atypical EAE symptoms were significantly ameliorated in mice that only expressed IFNGR in endothelial cells (EC-IFNGR mice). In these EC-IFNGR mice, inflammatory immune cell infiltration and demyelination were significantly inhibited in the brain but not in the spinal cord. In contrast to IFNGR-deficient mice, the functional integrity of the BBB was preserved in EC-IFNGR mice. This could be due to enhanced IFN-γ-induced claudin-5 expression that resulted in increased paracellular tightness of brain endothelial cell cultures (27). Therefore, these results indicate that endothelial expression of IFNGR is necessary for maintaining BBB function and preventing atypical EAE and brain inflammation. Further analysis must be performed in order to establish differential effects of IFN-γ on the BSCB.

IFN-γ AND CNS-RESIDENT CELLS

There are two groups of glial cells in the CNS: the macroglia, including astrocytes, oligodendrocytes, and ependymal cells, and the microglia. Several studies support the notion that glial cells not only provide functional support to neurons nor are they only a target of autoimmune injury, but are also active players in the development and progression of MS and EAE. Indeed, studies using an adoptive transfer EAE model have suggested that prevention of atypical EAE by IFN-γ is dependent on IFN-γ signaling not only in encephalitogenic T cells but also in glial cells (19, 25). Below, we will review the opposite effects of IFN-γ on glial cells and neurons, highlighting its less known protective functions and the mechanisms whereby IFN-γ is able to exert neuroprotection (**Figure 1**).

Oligodendrocytes

Oligodendrocytes are CNS-resident myelin-producing cells and thus necessary for remyelination. Loss of oligodendrocytes, demyelination, and axonal damage are hallmarks of MS and EAE. Several *in vitro* studies have shown detrimental effects of IFN-γ on oligodendrocyte survival (28). Consistently, overexpression of IFN-γ in the CNS results in oligodendrocyte apoptosis and inhibition of myelination during development and after a demye-linating insult (15, 16, 29). However, when exposed to low levels of IFN-γ, oligodendrocytes were protected against oxidative stress and showed enhanced proteasome activity, two important processes preventing the accumulation of oxidized proteins, mitochondrial dysfunction and apoptosis (30). Furthermore, transgenic mice expressing low levels of IFN-γ were protected against chemically induced demyelination with cuprizone, and these animals did not show signs of oligodendroglial death, astrogliosis, or microgliosis compared to wild-type mice exposed to the toxin (31). Interestingly, elevated levels of insulin-like growth factor (IGF)-1 were detected in the CNS of these transgenic mice, which might contribute to the observed protective effects of IFN-γ, as IGF-1 has been demonstrated to inhibit oligodendrocyte apoptosis and promote myelination (31). Therefore, these results indicate that IFN-γ dose is critical in determining the survival of oligodendrocytes.

Those studies performed with transgenic mice have the limitation that overexpression of IFN-γ was induced during myelin formation whereas EAE and MS occur when myelination has been established in adulthood. In order to overcome this pitfall, the same authors developed an elegant *in vivo* experimental model that allowed temporally regulated delivery of IFN-γ to the CNS through a tetracycline-controllable system. IFN-γ delivery in the CNS that was first detected in the acute phase of EAE ameliorated disease severity and prevented oligodendrocyte loss, demyelination, and axonal damage. By contrast, delivery of this cytokine during the EAE remitting phase delayed disease recovery and inhibited remyelination (32). Interestingly, the effects of this cytokine in oligodendrocytes were mediated by the activation of protein kinase RNA-like endoplasmic reticulum kinase (PERK)

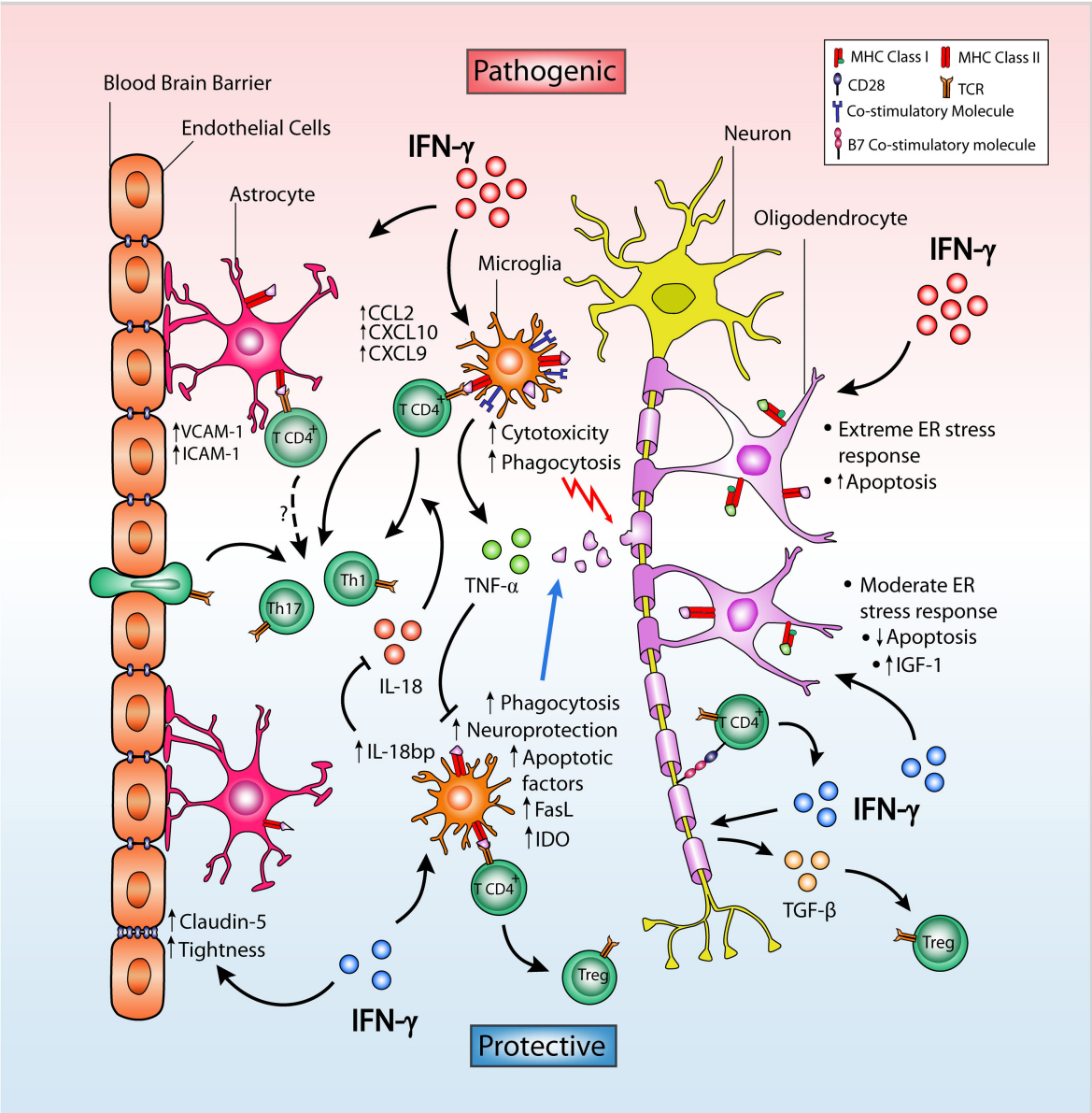

FIGURE 1 | Dual role of IFN-γ on CNS-resident cells in EAE. IFN-γ in the CNS may facilitate helper T cell infiltration and neuroinflammation by inducing expression of VCAM-1, ICAM-1, and the chemokines CCL2, CXCL9, and CXCL10 in astrocytes located in close proximity to the BBB. Despite this, IFN-γ promotes BBB integrity by enhancing the expression and membrane distribution of claudin-5, in turn increasing tightness and ameliorating EAE severity. In microglia, IFN-γ differentially causes pathogenic cytotoxicity or neuroprotection and apoptosis depending on dose. Low-dose IFN-γ can induce tolerogenic microglia, while high doses result in increased expression of MHC class II protein and co-stimulatory molecules, leading to encephalitogenic T cell activation. TNF-α expression is upregulated by IFN-γ in microglia and inhibits their neuroprotective properties. In contrast, IFN-γ upregulates microglial IDO expression limiting T cell proliferation and inducing Fas and FasL expression favoring increased T cell apoptosis. IFN-γ also induces IL-18bp expression in microglia which inhibits the proinflammatory actions of IL-18 inhibiting EAE. In neurons, IFN-γ can induce B7 co-stimulatory molecules and TGF-β expression, promoting a Treg cell population that suppresses EAE. In oligodendrocytes, the effect of IFN-γ depends on both dose and the level of ER stress. By inducing expression of MHC molecules and other immune proteins, IFN-γ overloads the stress response in actively myelinating oligodendrocytes causing cell death, whereas a moderate stress response induced by low dose of IFN-γ on steady-state oligodendrocytes decreases apoptosis and increases IGF-1 levels in the CNS promoting remyelination of post-neuroinflammatory damage.

and the phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2a), factors associated with the endoplasmic reticulum (ER) stress response (29, 32). The authors explained these apparently contradictory results proposing a dual role of IFN-γ on oligodendrocyte survival depending on their developmental stage and cellular stress (33, 34). According to this model, during the active process of myelination such as CNS development or remyelination (i.e., EAE remission), IFN-γ causes an overloaded ER stress response by increasing expression of MHC molecules and other inflammatory proteins in oligodendrocytes with already elevated

ER stress, resulting in apoptotic program activation. Conversely, mature oligodendrocytes in adult mice produce significantly lower levels of membrane to maintain myelin homeostasis (i.e., acute EAE). In this case, IFN-γ would induce a moderate and protective ER stress response leading to oligodendrocyte survival and remyelination (33–35). Further investigations have determined that protection of oligodendrocytes in EAE may be mediated through Janus kinase (JAK) and the transcription factor signal transducer and activator of transcription (STAT)-1 signaling, as overexpression of the suppressor of cytokine signal (SOCS)-1, a competitive inhibitor of the IFN-γ-induced JAK/STAT-1 signaling pathway resulted in early EAE onset, enhanced inflammation, and oligodendrocyte apoptosis (30). Instead, the signal transduction pathway activating the transcription factor interferon regulatory factor (IRF)-1 would play a proinflammatory role in oligodendrocytes during EAE progression (36).

Microglia

Microglia comprise 10–20% of all glial cells and are considered the immune sentinels of the CNS (37). They develop a broad and versatile range of functions involved in inflammation, immunomodulation, and promotion of neural repair that can be mediated in part by IFN-γ (38, 39). IFN-γ potentiates the phagocytic activity of microglia (40) and induces the expression of MHC class II and co-stimulatory molecules. This enables them to function as antigen-presenting cells (APCs) for infiltrating myelin-specific T cells leading to inflammation and demyelination (41–45).

During neuroinflammation, different subtypes of microglia can be distinguished according to their activation status. M1 microglia are primarily associated with an inflammatory phenotype and EAE initiation, while M2 microglia play an anti-inflammatory role and participate in tissue repair and remodeling associated with EAE recovery (5, 46). Despite being a potent activator of microglia and their polarization toward M1, IFN-γ can, at least in part, regulate the dual activity of microglia, modulating both pathogenic and regenerative processes (40, 47–49). How can IFN-γ lead these opposing roles?.

Evidence suggests that IFN-γ dose constitutes a fine-tune mechanism of regulation determining the balance between inflammatory and anti-inflammatory microglia. IFN-γ along with neuroantigen can determine an effector or regulatory helper T cell response modulating the activation state of microglia in a dose-dependent manner (48). Myelin oligodendrocyte glycoprotein (MOG)-specific $CD4^+$ T cells co-cultured with microglia that were pre-activated with a high dose of IFN-γ and MOG peptide were primarily differentiated toward $CD4^+CD25^+FoxP3^-$ effector T cells. Instead, microglia primed with a low dose of both IFN-γ and neuroantigen induced the expansion of stable $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) capable of suppressing EAE after adoptive transfer (48). Furthermore, low concentrations of IFN-γ enabled microglia to perform neuroprotective functions such as clearance of glutamate, neuronal survival, neurogenesis, and, to a lesser extent, oligodendrogenesis in response to CNS insult (50–52). Remarkably, EAE disease onset was significantly delayed in mice that were stereotaxically injected with microglia activated *in vitro* by a low dose of IFN-γ into the cerebral ventricles 7 days after immunization (53). By contrast, high concentrations of IFN-γ rendered microglia cytotoxic and impaired their neuroprotective activities (50–53). Interestingly, the dose-dependent paradoxical effects of IFN-γ may be mediated by microglial production of tumor necrosis factor (TNF)-α. Neutralization of TNF-α, whose expression is upregulated in IFN-γ-activated microglia, boosted both the neurogenesis and oligodendrogenesis induced by microglia activated with low-dose IFN-γ, whereas the addition of TNF-α to similar cell cultures prevented these cell renewal processes (52). Thus, low levels of IFN-γ induce beneficial effects on microglia, which are counteracted by the microglial production of TNF-α in response to increasing levels of IFN-γ.

On the other hand, IFN-γ can induce several self-limiting negative feedback mechanisms to restrain the magnitude and duration of its proinflammatory effects on microglia and to provide CNS protection. For example, IFN-γ induces mRNA expression of SOCS-1 which antagonizes IFN-γ-induced STAT-1 activation (54). Indeed, overexpression of SOCS-1 inhibited IFN-γ-induced expression of MHC class II and CD40 in microglia by inhibiting STAT-1-mediated expression of the class II transactivator (CIITA) transcription factor (54, 55). Furthermore, IFN-γ induced microglial cell death through the upregulation of apoptotic proteins, especially bcl-2-associated X protein (Bax), in EAE (56). This process was only observed in the advanced stage of EAE, suggesting a disease stage-specific role of IFN-γ on microglia in EAE, and likely MS. In fact, the activation and subsequent death of microglia induced by IFN-γ was proposed as a possible mechanism underlying MS relapse and remission (56).

Another important regulatory mechanism of IFN-γ is the suppression of T cell functions by localized catabolism of the aminoacid tryptophan, which is essential for cell growth and functioning. Indoleamine-2,3-dioxygenase (IDO), whose expression in microglia is upregulated by the IFN-γ-induced STAT-1 and phosphatidylinositol 3-kinase (PI3K) signaling pathways (57), catabolizes tryptophan to kynurenine, and has been shown to play a protective role in EAE (58). Microglial IDO expression, induced by IFN-γ, reduced extracellular tryptophan and increased kynurenine which suppressed the proliferation of myelin-specific T cells and inhibited production of proinflammatory Th1 cytokines (59). In addition, tryptophan deprivation made T cells more susceptible to apoptosis through Fas-Fas ligand (FasL)-mediated signaling (60), whose expression can be induced in microglia by IFN-γ (61), enabling them with an additional protective mechanism by facilitating apoptosis of myelin-specific T cells.

Additionally, IFN-γ establishes a regulatory feedback limiting the inflammatory activity of interleukin (IL)-18, a member of the IL-1 family produced by microglia that synergizes with IL-12 to induce Th1 polarization and IFN-γ production. IL-18 is upregulated during EAE (62) and its neutralization inhibited EAE (63). In turn, IFN-γ induced the expression of IL-18 binding protein (IL-18bp), an endogenous inhibitor of IL-18, in CNS-resident microglia and infiltrating macrophages during EAE (64). Interestingly, augmented IL-18bp expression in the CNS mediated by an adenoviral vector, blocked the induction of Th17 cells, but not Th1 cells, in the CNS and significantly reduced the incidence and severity of EAE (64). Therefore, these results

suggest that IFN-γ-dependent IL-18bp production in microglia might regulate the balance between the Th1 and Th17 responses during autoimmune neuroinflammation.

Astrocytes

Astrocytes are the most abundant glial cell population and are essential for brain homeostasis and neuronal function. They play a major role in maintaining both the structure and functional integrity of the BBB, develop metabolic functions, and seclude damaged areas in the CNS. Astrocytes also exhibit a variety of immune functions whereby they may both stimulate and restrain neuroinflammation (65–68). Although the capability of astrocytes to function as APCs *in vivo* is still controversial, some investigators have identified astrocytes expressing MHC class II molecules (69), co-stimulatory B7 molecules (70), and intercellular adhesion molecule (ICAM)-1 (71) at the edges of active MS lesions. The expression of these antigen-presenting molecules in astrocytes is upregulated by IFN-γ both *in vivo* and *in vitro* (12). In line with these results, astrocytes exposed to IFN-γ *in vitro* can induce proliferation of myelin-specific T cells and Th1 differentiation (66, 68). Importantly, $CD4^+$ T cells activated by astrocytes treated with IFN-γ and pulsed with myelin protein were able to induce EAE by adoptive transfer (72). In addition, IFN-γ upregulated expression of ICAM-1 and vascular cell adhesion molecule (VCAM)-1 on primary astrocytes (72, 73), which might have a direct impact on the extravasation of T cells into the CNS considering the close apposition of astrocyte foot processes to the microvascular endothelium of the BBB.

IFN-γ signaling in astrocytes also plays an important role in triggering expression of a wide range of chemokines involved in the recruitment of inflammatory cells to the CNS in EAE and MS. At the edges of demyelinating MS lesions, astrocytes expressed CCL2, CXCL10, and their respective receptors CCR2 and CXCR3 (74, 75). In EAE, CCL2 and CXCL10 were localized predominantly in astrocytes surrounding inflammatory lesions (76–78) and their expression was abolished in IFNGR-deficient mice (76). *In vitro* studies have confirmed that both human and murine astrocytes are induced by IFN-γ to produce CCL2, CXCL9, and CXCL10 (79–81).

Selective silencing of IFN-γ signaling in astrocytes using a lentiviral vector significantly ameliorated both actively and passively induced EAE (82). Surprisingly, purified astrocytes from spinal cord of these animals not only exhibited reduced expression of IFN-γ-inducible chemokines such as CXCL9, CXCL10, and CXCL11 but also inhibited expression of chemokines induced by IL-17 signaling such as CXCL1, CXCL2, and CCL20 (82). These results were confirmed *in vitro* by analyzing astrocytes deficient in IFNGR and suggest that IFN-γ signaling is the common pathway in both Th1 and Th17 cell-mediated EAE (82). In contrast to results depicted in astrocytes, the authors of this study found that selective blocking of IFN-γ signaling in microglia resulted in more severe EAE progression that was associated with significantly enhanced CNS inflammatory infiltration. In addition, IFN-γ signaling restricted microglia proliferation (82). Together, these results suggest that IFN-γ would exert opposing roles on two different CNS-resident cell types in EAE: inflammatory on astrocytes and protective on microglia.

However, another study using transgenic mice expressing a signaling deficient dominant-negative IFNGR1 specifically on astrocytes reached opposite conclusions when analyzing EAE progression (83). Compared to wild-type mice, these animals did not show alteration in the incidence, disease onset, or initiation of clinical symptoms. Instead, during the transition from acute to chronic disease, the clinical score and mortality were significantly increased, suggesting that IFN-γ signaling in astrocytes provides disease stage-specific protection (83). Different methodological strategies used to ablate IFN-γ signaling might explain the discrepant results found between these two studies (82, 83). A posterior study demonstrated that persistent CNS inflammation and progressive disability observed in these mice lacking IFN-γ signaling in astrocytes was associated with elevated expression of IL-6 and sustained proliferation and activation of microglia (84). Taken together, these results indicate that IFN-γ signaling plays an important role in controlling the proliferation of microglia during EAE either directly on microglia (82) or indirectly through astrocytes, (84) and highlight the crucial role of IFN-γ in the signaling network between astrocytes and microglia during EAE pathogenesis.

Neurons

There is discrepant evidence regarding the relationship between neurons and IFN-γ, mainly described in the non-inflammatory brain, with both protective and detrimental effects (3, 9, 85, 86). In EAE, it has been observed that treatment with nerve growth factor (NGF), a well-known neural factor involved in neuron survival, decreased disease severity, and increased IFNGR expression in spinal cord neurons (87). Even more, neurons were induced to express transforming growth factor (TGF)-β and B7 molecules in response to IFN-γ produced by encephalitogenic T cells. Remarkably, encephalitogenic T cell–neuron interaction induced higher neuron survival and promoted the conversion of encephalitogenic $CD4^+$ T cells to functional $CD4^+CD25^+TGF\text{-}\beta^+CTLA\text{-}4^+$ Tregs. The adoptive transfer of these converted Tregs suppressed EAE. Strikingly, the acquisition of this regulatory phenotype occurred in the CNS and was mediated by the production of IFN-γ and TNF-α by T cells (88).

CONCLUDING REMARKS

Despite several studies having reported an inflammatory effect of IFN-γ in the CNS during EAE development, accumulating data have also demonstrated that IFN-γ possesses neuroprotective activity in this disease. The recent evidence summarized in this review underscores the duality of IFN-γ on CNS-resident cells in EAE and provides different mechanisms whereby this cytokine exerts these opposing activities. In low doses, IFN-γ induces protection in both microglia and oligodendrocytes, while in high doses it induces disease worsening effects in both glial cell types. In astrocytes, the evidence reveals a primarily disease-promoting role for IFN-γ signaling, although it may be disease stage-specific. Interestingly, IFN-γ also exerts opposing roles in oligodendrocytes depending on the cell maturation status and cellular stress response. Importantly, these findings implicate that defects or fluctuations related to the expression of IFN-γ, its receptor, and/

or its signaling pathway may underlie the immunopathogenesis of MS as well as other demyelinating inflammatory diseases. Therefore, delineating the precise role of IFN-γ in the CNS and on CNS-resident cells might provide the basis for fine-tuning the development of CNS-targeted selective therapy.

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REFERENCES

1. Nylander A, Hafler DA. Multiple sclerosis. *J Clin Invest* (2012) **122**(4):1180–8. doi:10.1172/JCI58649
2. Simmons SB, Pierson ER, Lee SY, Goverman JM. Modeling the heterogeneity of multiple sclerosis in animals. *Trends Immunol* (2013) **34**(8):410–22. doi:10.1016/j.it.2013.04.006
3. Olsson T. Cytokines in neuroinflammatory disease: role of myelin autoreactive T cell production of interferon-gamma. *J Neuroimmunol* (1992) **40**(2–3):211–8. doi:10.1016/0165-5728(92)90135-8
4. Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KH. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. *Clin Exp Immunol* (2010) **162**(1):1–11. doi:10.1111/j.1365-2249.2010.04143.x
5. Duffy SS, Lees JG, Moalem-Taylor G. The contribution of immune and glial cell types in experimental autoimmune encephalomyelitis and multiple sclerosis. *Mult Scler Int* (2014) **2014**:285245. doi:10.1155/2014/285245
6. Sanvito L, Constantinescu CS, Gran B, 't Hart BA. The multifaceted role of interferon. *Open Autoimmun J* (2010) **2**:151–9. doi:10.2174/1876894601002040151
7. Kelchtermans H, Billiau A, Matthys P. How interferon-gamma keeps autoimmune diseases in check. *Trends Immunol* (2008) **29**(10):479–86. doi:10.1016/j.it.2008.07.002
8. Arellano G, Ottum P, Reyes L, Burgos P, Naves R. Stage-specific role of interferon-gamma in experimental autoimmune encephalomyelitis and multiple sclerosis. *Front Immunol* (2015) **6**:492. doi:10.3389/fimmu.2015.00492
9. Popko B, Corbin JG, Baerwald KD, Dupree J, Garcia AM. The effects of interferon-gamma on the central nervous system. *Mol Neurobiol* (1997) **14**(1–2):19–35. doi:10.1007/BF02740619
10. Simmons RD, Willenborg DO. Direct injection of cytokines into the spinal cord causes autoimmune encephalomyelitis-like inflammation. *J Neurol Sci* (1990) **100**(1–2):37–42. doi:10.1016/0022-510X(90)90010-K
11. Sethna MP, Lampson LA. Immune modulation within the brain: recruitment of inflammatory cells and increased major histocompatibility antigen expression following intracerebral injection of interferon-gamma. *J Neuroimmunol* (1991) **34**(2–3):121–32. doi:10.1016/0165-5728(91)90121-M
12. Vass K, Lassmann H. Intrathecal application of interferon gamma. Progressive appearance of MHC antigens within the rat nervous system. *Am J Pathol* (1990) **137**(4):789–800.
13. Vass K, Heininger K, Schafer B, Linington C, Lassmann H. Interferon-gamma potentiates antibody-mediated demyelination in vivo. *Ann Neurol* (1992) **32**(2):198–206. doi:10.1002/ana.410320212
14. Renno T, Taupin V, Bourbonniere L, Verge G, Tran E, De Simone R, et al. Interferon-gamma in progression to chronic demyelination and neurological deficit following acute EAE. *Mol Cell Neurosci* (1998) **12**(6):376–89. doi:10.1006/mcne.1998.0725
15. Corbin JG, Kelly D, Rath EM, Baerwald KD, Suzuki K, Popko B. Targeted CNS expression of interferon-gamma in transgenic mice leads to hypomyelination, reactive gliosis, and abnormal cerebellar development. *Mol Cell Neurosci* (1996) **7**(5):354–70. doi:10.1006/mcne.1996.0026
16. Horwitz MS, Evans CF, McGavern DB, Rodriguez M, Oldstone MB. Primary demyelination in transgenic mice expressing interferon-gamma. *Nat Med* (1997) **3**(9):1037–41. doi:10.1038/nm0997-1037
17. Voorthuis JA, Uitdehaag BM, De Groot CJ, Goede PH, van der Meide PH, Dijkstra CD. Suppression of experimental allergic encephalomyelitis by intraventricular administration of interferon-gamma in Lewis rats. *Clin Exp Immunol* (1990) **81**(2):183–8. doi:10.1111/j.1365-2249.1990.tb03315.x
18. Heremans H, Dillen C, Groenen M, Martens E, Billiau A. Chronic relapsing experimental autoimmune encephalomyelitis (CREAE) in mice: enhancement by monoclonal antibodies against interferon-gamma. *Eur J Immunol* (1996) **26**(10):2393–8. doi:10.1002/eji.1830261019
19. Naves R, Singh SP, Cashman KS, Rowse AL, Axtell RC, Steinman L, et al. The interdependent, overlapping, and differential roles of type I and II IFNs in the pathogenesis of experimental autoimmune encephalomyelitis. *J Immunol* (2013) **191**(6):2967–77. doi:10.4049/jimmunol.1300419
20. Billiau A, Heremans H, Vandekerckhove F, Dijkmans R, Sobis H, Meulepas E, et al. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J Immunol* (1988) **140**(5):1506–10.
21. Pierson E, Simmons SB, Castelli L, Goverman JM. Mechanisms regulating regional localization of inflammation during CNS autoimmunity. *Immunol Rev* (2012) **248**(1):205–15. doi:10.1111/j.1600-065X.2012.01126.x
22. Abromson-Leeman S, Bronson R, Luo Y, Berman M, Leeman R, Leeman J, et al. T-cell properties determine disease site, clinical presentation, and cellular pathology of experimental autoimmune encephalomyelitis. *Am J Pathol* (2004) **165**(5):1519–33. doi:10.1016/S0002-9440(10)63410-4
23. Wensky AK, Furtado GC, Marcondes MC, Chen S, Manfra D, Lira SA, et al. IFN-gamma determines distinct clinical outcomes in autoimmune encephalomyelitis. *J Immunol* (2005) **174**(3):1416–23. doi:10.4049/jimmunol.174.3.1416
24. Mangalam AK, Luo N, Luckey D, Papke L, Hubbard A, Wussow A, et al. Absence of IFN-gamma increases brain pathology in experimental autoimmune encephalomyelitis-susceptible DRB1*0301.DQ8 HLA transgenic mice through secretion of proinflammatory cytokine IL-17 and induction of pathogenic monocytes/microglia into the central nervous system. *J Immunol* (2014) **193**(10):4859–70. doi:10.4049/jimmunol.1302008
25. Lees JR, Golumbek PT, Sim J, Dorsey D, Russell JH. Regional CNS responses to IFN-gamma determine lesion localization patterns during EAE pathogenesis. *J Exp Med* (2008) **205**(11):2633–42. doi:10.1084/jem.20080155
26. Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med* (2008) **14**(3):337–42. doi:10.1038/nm1715
27. Ni C, Wang C, Zhang J, Qu L, Liu X, Lu Y, et al. Interferon-gamma safeguards blood-brain barrier during experimental autoimmune encephalomyelitis. *Am J Pathol* (2014) **184**(12):3308–20. doi:10.1016/j.ajpath.2014.08.019
28. Pouly S, Becher B, Blain M, Antel JP. Interferon-gamma modulates human oligodendrocyte susceptibility to Fas-mediated apoptosis. *J Neuropathol Exp Neurol* (2000) **59**(4):280–6.
29. Lin W, Harding HP, Ron D, Popko B. Endoplasmic reticulum stress modulates the response of myelinating oligodendrocytes to the immune cytokine interferon-gamma. *J Cell Biol* (2005) **169**(4):603–12. doi:10.1083/jcb.200502086
30. Balabanov R, Strand K, Goswami R, McMahon E, Begolka W, Miller SD, et al. Interferon-gamma-oligodendrocyte interactions in the regulation of experimental autoimmune encephalomyelitis. *J Neurosci* (2007) **27**(8):2013–24. doi:10.1523/JNEUROSCI.4689-06.2007
31. Gao X, Gillig TA, Ye P, D'Ercole AJ, Matsushima GK, Popko B. Interferon-gamma protects against cuprizone-induced demyelination. *Mol Cell Neurosci* (2000) **16**(4):338–49. doi:10.1006/mcne.2000.0883
32. Lin W, Bailey SL, Ho H, Harding HP, Ron D, Miller SD, et al. The integrated stress response prevents demyelination by protecting oligodendrocytes against immune-mediated damage. *J Clin Invest* (2007) **117**(2):448–56. doi:10.1172/JCI29571
33. Lin W, Kemper A, Dupree JL, Harding HP, Ron D, Popko B. Interferon-gamma inhibits central nervous system remyelination through a process modulated by endoplasmic reticulum stress. *Brain* (2006) **129**(Pt 5):1306–18. doi:10.1093/brain/awl044
34. Lin W, Popko B. Endoplasmic reticulum stress in disorders of myelinating cells. *Nat Neurosci* (2009) **12**(4):379–85. doi:10.1038/nn.2273
35. Lees JR, Cross AH. A little stress is good: IFN-gamma, demyelination, and multiple sclerosis. *J Clin Invest* (2007) **117**(2):297–9. doi:10.1172/JCI31254
36. Ren Z, Wang Y, Tao D, Liebenson D, Liggett T, Goswami R, et al. Overexpression of the dominant-negative form of interferon regulatory factor 1 in oligodendrocytes protects against experimental autoimmune encephalomyelitis. *J Neurosci* (2011) **31**(23):8329–41. doi:10.1523/JNEUROSCI.1028-11.2011

37. Schwartz M, Butovsky O, Bruck W, Hanisch UK. Microglial phenotype: is the commitment reversible? *Trends Neurosci* (2006) **29**(2):68–74. doi:10.1016/j.tins.2005.12.005
38. Benveniste EN. Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis. *J Mol Med* (1997) **75**(3):165–73. doi:10.1007/s001090050101
39. Aloisi F. Immune function of microglia. *Glia* (2001) **36**(2):165–79. doi:10.1002/glia.1106
40. Merson TD, Binder MD, Kilpatrick TJ. Role of cytokines as mediators and regulators of microglial activity in inflammatory demyelination of the CNS. *Neuromolecular Med* (2010) **12**(2):99–132. doi:10.1007/s12017-010-8112-z
41. Wong GH, Bartlett PF, Clark-Lewis I, Battye F, Schrader JW. Inducible expression of H-2 and Ia antigens on brain cells. *Nature* (1984) **310**(5979):688–91. doi:10.1038/310688a0
42. Suzumura A, Mezitis SG, Gonatas NK, Silberberg DH. MHC antigen expression on bulk isolated macrophage-microglia from newborn mouse brain: induction of Ia antigen expression by gamma-interferon. *J Neuroimmunol* (1987) **15**(3):263–78. doi:10.1016/0165-5728(87)90121-4
43. Menendez Iglesias B, Cerase J, Ceracchini C, Levi G, Aloisi F. Analysis of B7-1 and B7-2 costimulatory ligands in cultured mouse microglia: upregulation by interferon-gamma and lipopolysaccharide and downregulation by interleukin-10, prostaglandin E2 and cyclic AMP-elevating agents. *J Neuroimmunol* (1997) **72**(1):83–93. doi:10.1016/S0165-5728(96)00155-5
44. Murphy AC, Lalor SJ, Lynch MA, Mills KH. Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. *Brain Behav Immun* (2010) **24**(4):641–51. doi:10.1016/j.bbi.2010.01.014
45. Matsumoto Y, Ohmori K, Fujiwara M. Immune regulation by brain cells in the central nervous system: microglia but not astrocytes present myelin basic protein to encephalitogenic T cells under in vivo-mimicking conditions. *Immunology* (1992) **76**(2):209–16.
46. Goldmann T, Prinz M. Role of microglia in CNS autoimmunity. *Clin Dev Immunol* (2013) **2013**:208093. doi:10.1155/2013/208093
47. Bruck W, Porada P, Poser S, Rieckmann P, Hanefeld F, Kretzschmar HA, et al. Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Ann Neurol* (1995) **38**(5):788–96. doi:10.1002/ana.410380514
48. Ebner F, Brandt C, Thiele P, Richter D, Schliesser U, Siffrin V, et al. Microglial activation milieu controls regulatory T cell responses. *J Immunol* (2013) **191**(11):5594–602. doi:10.4049/jimmunol.1203331
49. Prineas JW, Graham JS. Multiple sclerosis: capping of surface immunoglobulin G on macrophages engaged in myelin breakdown. *Ann Neurol* (1981) **10**(2):149–58. doi:10.1002/ana.410100205
50. Shaked I, Tchoresh D, Gersner R, Meiri G, Mordechai S, Xiao X, et al. Protective autoimmunity: interferon-gamma enables microglia to remove glutamate without evoking inflammatory mediators. *J Neurochem* (2005) **92**(5):997–1009. doi:10.1111/j.1471-4159.2004.02954.x
51. Butovsky O, Talpalar AE, Ben-Yaakov K, Schwartz M. Activation of microglia by aggregated beta-amyloid or lipopolysaccharide impairs MHC-II expression and renders them cytotoxic whereas IFN-gamma and IL-4 render them protective. *Mol Cell Neurosci* (2005) **29**(3):381–93. doi:10.1016/j.mcn.2005.03.005
52. Butovsky O, Ziv Y, Schwartz A, Landa G, Talpalar AE, Pluchino S, et al. Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. *Mol Cell Neurosci* (2006) **31**(1):149–60. doi:10.1016/j.mcn.2005.10.006
53. Butovsky O, Landa G, Kunis G, Ziv Y, Avidan H, Greenberg N, et al. Induction and blockage of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis. *J Clin Invest* (2006) **116**(4):905–15. doi:10.1172/JCI26836
54. Wesemann DR, Dong Y, O'Keefe GM, Nguyen VT, Benveniste EN. Suppressor of cytokine signaling 1 inhibits cytokine induction of CD40 expression in macrophages. *J Immunol* (2002) **169**(5):2354–60. doi:10.4049/jimmunol.169.5.2354
55. O'Keefe GM, Nguyen VT, Ping Tang LL, Benveniste EN. IFN-gamma regulation of class II transactivator promoter IV in macrophages and microglia: involvement of the suppressors of cytokine signaling-1 protein. *J Immunol* (2001) **166**(4):2260–9. doi:10.4049/jimmunol.166.4.2260
56. Takeuchi H, Wang J, Kawanokuchi J, Mitsuma N, Mizuno T, Suzumura A. Interferon-gamma induces microglial-activation-induced cell death: a hypothetical mechanism of relapse and remission in multiple sclerosis. *Neurobiol Dis* (2006) **22**(1):33–9. doi:10.1016/j.nbd.2005.09.014
57. Yadav MC, Burudi EM, Alirezaei M, Flynn CC, Watry DD, Lanigan CM, et al. IFN-gamma-induced IDO and WRS expression in microglia is differentially regulated by IL-4. *Glia* (2007) **55**(13):1385–96. doi:10.1002/glia.20544
58. Kwidzinski E, Bunse J, Aktas O, Richter D, Mutlu L, Zipp F, et al. Indolamine 2,3-dioxygenase is expressed in the CNS and down-regulates autoimmune inflammation. *FASEB J* (2005) **19**(10):1347–9. doi:10.1096/fj.04-3228fje
59. Platten M, Ho PP, Youssef S, Fontoura P, Garren H, Hur EM, et al. Treatment of autoimmune neuroinflammation with a synthetic tryptophan metabolite. *Science* (2005) **310**(5749):850–5. doi:10.1126/science.1117634
60. Lee GK, Park HJ, Macleod M, Chandler P, Munn DH, Mellor AL. Tryptophan deprivation sensitizes activated T cells to apoptosis prior to cell division. *Immunology* (2002) **107**(4):452–60. doi:10.1046/j.1365-2567.2002.01526.x
61. Badie B, Schartner J, Vorpahl J, Preston K. Interferon-gamma induces apoptosis and augments the expression of Fas and Fas ligand by microglia in vitro. *Exp Neurol* (2000) **162**(2):290–6. doi:10.1006/exnr.1999.7345
62. Jander S, Stoll G. Differential induction of interleukin-12, interleukin-18, and interleukin-1beta converting enzyme mRNA in experimental autoimmune encephalomyelitis of the Lewis rat. *J Neuroimmunol* (1998) **91**(1–2):93–9. doi:10.1016/S0165-5728(98)00162-3
63. Wildbaum G, Youssef S, Grabie N, Karin N. Neutralizing antibodies to IFN-gamma-inducing factor prevent experimental autoimmune encephalomyelitis. *J Immunol* (1998) **161**(11):6368–74.
64. Millward JM, Lobner M, Wheeler RD, Owens T. Inflammation in the central nervous system and Th17 responses are inhibited by IFN-gamma-Induced IL-18 binding protein. *J Immunol* (2010) **185**(4):2458–66. doi:10.4049/jimmunol.0902153
65. Miljkovic D, Timotijevic G, Mostarica Stojkovic M. Astrocytes in the tempest of multiple sclerosis. *FEBS Lett* (2011) **585**(23):3781–8. doi:10.1016/j.febslet.2011.03.047
66. Chastain EM, Duncan DS, Rodgers JM, Miller SD. The role of antigen presenting cells in multiple sclerosis. *Biochim Biophys Acta* (2011) **1812**(2):265–74. doi:10.1016/j.bbadis.2010.07.008
67. Nair A, Frederick TJ, Miller SD. Astrocytes in multiple sclerosis: a product of their environment. *Cell Mol Life Sci* (2008) **65**(17):2702–20. doi:10.1007/s00018-008-8059-5
68. Dong Y, Benveniste EN. Immune function of astrocytes. *Glia* (2001) **36**(2):180–90. doi:10.1002/glia.1107
69. Zeinstra E, Wilczak N, Streefland C, De Keyser J. Astrocytes in chronic active multiple sclerosis plaques express MHC class II molecules. *Neuroreport* (2000) **11**(1):89–91. doi:10.1097/00001756-200001170-00018
70. Zeinstra E, Wilczak N, De Keyser J. Reactive astrocytes in chronic active lesions of multiple sclerosis express co-stimulatory molecules B7-1 and B7-2. *J Neuroimmunol* (2003) **135**(1–2):166–71. doi:10.1016/S0165-5728(02)00462-9
71. Sobel RA, Mitchell ME, Fondren G. Intercellular adhesion molecule-1 (ICAM-1) in cellular immune reactions in the human central nervous system. *Am J Pathol* (1990) **136**(6):1309–16.
72. Tan L, Gordon KB, Mueller JP, Matis LA, Miller SD. Presentation of proteolipid protein epitopes and B7-1-dependent activation of encephalitogenic T cells by IFN-gamma-activated SJL/J astrocytes. *J Immunol* (1998) **160**(9):4271–9.
73. Rosenman SJ, Shrikant P, Dubb L, Benveniste EN, Ransohoff RM. Cytokine-induced expression of vascular cell adhesion molecule-1 (VCAM-1) by astrocytes and astrocytoma cell lines. *J Immunol* (1995) **154**(4):1888–99.
74. Van Der Voorn P, Tekstra J, Beelen RH, Tensen CP, Van Der Valk P, De Groot CJ. Expression of MCP-1 by reactive astrocytes in demyelinating multiple sclerosis lesions. *Am J Pathol* (1999) **154**(1):45–51. doi:10.1016/S0002-9440(10)65249-2
75. Tanuma N, Shin T, Kogure K, Matsumoto Y. Differential role of TNF-alpha and IFN-gamma in the brain of rats with chronic relapsing autoimmune encephalomyelitis. *J Neuroimmunol* (1999) **96**(1):73–9. doi:10.1016/S0165-5728(99)00018-1
76. Carter SL, Muller M, Manders PM, Campbell IL. Induction of the genes for Cxcl9 and Cxcl10 is dependent on IFN-gamma but shows differential cellular expression in experimental autoimmune encephalomyelitis and by astrocytes and microglia in vitro. *Glia* (2007) **55**(16):1728–39. doi:10.1002/glia.20587

77. Glabinski AR, Tani M, Strieter RM, Tuohy VK, Ransohoff RM. Synchronous synthesis of alpha- and beta-chemokines by cells of diverse lineage in the central nervous system of mice with relapses of chronic experimental autoimmune encephalomyelitis. *Am J Pathol* (1997) **150**(2):617–30.
78. Ransohoff RM, Hamilton TA, Tani M, Stoler MH, Shick HE, Major JA, et al. Astrocyte expression of mRNA encoding cytokines IP-10 and JE/MCP-1 in experimental autoimmune encephalomyelitis. *FASEB J* (1993) **7**(6):592–600.
79. Salmaggi A, Gelati M, Dufour A, Corsini E, Pagano S, Baccalini R, et al. Expression and modulation of IFN-gamma-inducible chemokines (IP-10, Mig, and I-TAC) in human brain endothelium and astrocytes: possible relevance for the immune invasion of the central nervous system and the pathogenesis of multiple sclerosis. *J Interferon Cytokine Res* (2002) **22**(6):631–40. doi:10.1089/10799900260100114
80. Hayashi M, Luo Y, Laning J, Strieter RM, Dorf ME. Production and function of monocyte chemoattractant protein-1 and other beta-chemokines in murine glial cells. *J Neuroimmunol* (1995) **60**(1–2):143–50. doi:10.1016/0165-5728(95)00064-9
81. Zhou ZH, Chaturvedi P, Han YL, Aras S, Li YS, Kolattukudy PE, et al. IFN-gamma induction of the human monocyte chemoattractant protein (hMCP)-1 gene in astrocytoma cells: functional interaction between an IFN-gamma-activated site and a GC-rich element. *J Immunol* (1998) **160**(8):3908–16.
82. Ding X, Yan Y, Li X, Li K, Ciric B, Yang J, et al. Silencing IFN-gamma binding/signaling in astrocytes versus microglia leads to opposite effects on central nervous system autoimmunity. *J Immunol* (2015) **194**(9):4251–64. doi:10.4049/jimmunol.1303321
83. Hindinger C, Bergmann CC, Hinton DR, Phares TW, Parra GI, Hussain S, et al. IFN-gamma signaling to astrocytes protects from autoimmune mediated neurological disability. *PLoS One* (2012) **7**(7):e42088. doi:10.1371/journal.pone.0042088
84. Savarin C, Hinton DR, Valentin-Torres A, Chen Z, Trapp BD, Bergmann CC, et al. Astrocyte response to IFN-gamma limits IL-6-mediated microglia activation and progressive autoimmune encephalomyelitis. *J Neuroinflammation* (2015) **12**(1):79. doi:10.1186/s12974-015-0293-9
85. Gonzalez-Perez O, Jauregui-Huerta F, Galvez-Contreras AY. Immune system modulates the function of adult neural stem cells. *Curr Immunol Rev* (2010) **6**(3):167–73. doi:10.2174/157339510791823772
86. Hammarberg H, Lidman O, Lundberg C, Eltayeb SY, Gielen AW, Muhallab S, et al. Neuroprotection by encephalomyelitis: rescue of mechanically injured neurons and neurotrophin production by CNS-infiltrating T and natural killer cells. *J Neurosci* (2000) **20**(14):5283–91.
87. Stampachiacchiere B, Aloe L. Differential modulatory effect of NGF on MHC class I and class II expression in spinal cord cells of EAE rats. *J Neuroimmunol* (2005) **169**(1–2):20–30. doi:10.1016/j.jneuroim.2005.07.022
88. Liu Y, Teige I, Birnir B, Issazadeh-Navikas S. Neuron-mediated generation of regulatory T cells from encephalitogenic T cells suppresses EAE. *Nat Med* (2006) **12**(5):518–25. doi:10.1038/nm1402

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Stage-specific role of interferon-gamma in experimental autoimmune encephalomyelitis and multiple sclerosis

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The role of interferon (IFN)-γ in multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), has remained as an enigmatic paradox for more than 30 years. Several studies attribute this cytokine a prominent proinflammatory and pathogenic function in these pathologies. However, accumulating evidence shows that IFN-γ also plays a protective role inducing regulatory cell activity and modulating the effector T cell response. Several innate and adaptive immune cells also develop opposite functions strongly associated with the production of IFN-γ in EAE. Even the suppressive activity of different types of regulatory cells is dependent on IFN-γ. Interestingly, recent data supports a stage-specific participation of IFN-γ in EAE providing a plausible explanation for previous conflicting results. In this review, we will summarize and discuss such literature, emphasizing the protective role of IFN-γ on immune cells. These findings are fundamental to understand the complex role of IFN-γ in the pathogenesis of these diseases and can provide basis for potential stage-specific therapy for MS targeting IFN-γ-signaling or IFN-γ-producing immune cells.

Keywords: interferon-gamma, experimental autoimmune encephalomyelitis, multiple sclerosis, innate immunity, adaptive immunity, neuroinflammation

Introduction

Interferon (IFN)-γ is the only type II IFN family member. It is secreted by activated immune cells, mainly T and natural killer (NK) cells, but also B cells, NKT cells, and professional antigen presenting cells (APC). IFN-γ binds to a heterodimeric receptor, IFNGR, expressed ubiquitously on almost all cell types. Given its pleiotropic functions, IFN-γ plays a pivotal role in orchestrating immune system homeostasis (1–4). Historically, IFN-γ production has been considered the hallmark of T helper (Th)1 cells driving inflammation and autoimmunity, such as multiple sclerosis (MS). MS is an inflammatory and demyelinating disorder of the central nervous system (CNS) and is the leading cause of non-traumatic neurological disability in young adults (5). According to the clinical course, MS can be classified in different types: relapsing-remitting disease (RRMS), consisting of acute recurrent attacks followed by a variable degree of recovery, and progressive forms characterized by chronic and irreversible neurological disability (6).

To date, experimental autoimmune encephalomyelitis (EAE) remains as the animal model most widely used to study the immunopathological mechanisms and therapeutic approaches to MS (7, 8). EAE is induced by immunization with myelin-derived antigens in adjuvant or by the adoptive transfer of activated myelin-specific T cells into syngeneic naive hosts. First, an initiation/inductive phase occurs, where innate and adaptive immune cells are antigen stimulated in the periphery. That is followed by the effector phase characterized by an acute immune cell infiltration into the CNS, and a later chronic phase of inflammation and axonal damage (9).

Discrepant results have been reported in relation to the role of IFN-γ in EAE and MS (3, 4, 10). Factors such as dose, site specificity, and timing of action as well as interaction with other cytokines and cells can determine the net effect of IFN-γ (3, 10, Ottum et al., in preparation). Recent evidence supports a, not mutually exclusive, stage-specific role of IFN-γ in EAE providing an explanation to these controversial results and a model whereby this cytokine can both promote and limit the development of these pathologies. In this same Research Topic, we have reviewed the opposing roles of IFN-γ on CNS-resident cells in EAE and MS (Ottum et al., in preparation). Here, we will review the evidence on IFN-γ's dual role in the cells of the immune system in these same pathologies.

Two-Faced Role of IFN-γ in EAE and MS

Initially, a positive association between increased levels of IFN-γ and demyelinating lesions in the CNS in MS and EAE attributed this cytokine a pathological role (11–15). In mice, passive immunization of healthy animals with encephalitogenic Th1 lymphocytes producing IFN-γ was sufficiently capable of inducing EAE (16). Besides, mice deficient in T-bet, a transcription factor that drives Th1 differentiation, were protected from developing EAE (17, 18). The proinflammatory effects of IFN-γ were confirmed in a pilot clinical study showing that seven of eighteen RRMS patients treated with IFN-γ exhibited symptom exacerbations (19). Consistently, secondary progressive MS patients (SPMS) treated with antibodies against IFN-γ exhibited slightly reduced clinical symptoms (20).

However, subsequent studies have challenged the notion that IFN-γ is pathogenic, and there is accumulating evidence proposing a protective role for IFN-γ in EAE and MS. Systemic or intraventricular injection of IFN-γ in EAE mice reduced the severity of disease symptoms, morbidity, and mortality (21, 22), and systemic IFN-γ treatment in chronic-relapsing EAE (CREAE) significantly delayed the appearance of relapses (23). Likewise, anti-IFN-γ therapy exacerbated EAE symptoms and made a mice strain resistant to EAE susceptible to developing disease (21–26). These results have been corroborated using animals deficient in the IFN-γ gene, which showed increased incidence of EAE, earlier disease onset and more severe symptoms compared with control mice (27–29). Even more, animals lacking IFNGR developed EAE with higher susceptibility, severity, and lethality (30–32). Passive transfer of encephalitogenic splenic cells from EAE-induced IFNGR-deficient mice into either wild-type (WT) or IFNGR-deficient recipient mice led to the development of EAE, but only WT mice recovered from illness (33). Interestingly, in tumor necrosis factor (TNF)-α receptor-deficient mice, a higher frequency of Th1 cells and enhanced mRNA expression of IFN-γ in the CNS was associated with a milder EAE (34).

Finally, in the marmoset EAE model, administration of human IFN-γ did not aggravate clinical symptoms, and by contrast, there was a trend to delay the appearance of the neurological episodes associated with less inflammation and demyelination during the EAE late phase (35). Regarding MS, induction of endogenous IFNs production in progressive MS patients showed that some patients with improving symptoms had high levels of serum IFN-γ, while clinical worsening was related with low serum IFN-γ levels (36).

Stage-Specific Role of IFN-γ

The opposing activities that IFN-γ has in MS and the different models of EAE remain unresolved. However, collective evidence has shown that these paradoxical functions likely reflect a disease stage-specific opposing role of IFN-γ in EAE: promoting pathogenesis during the initiation phase but immunosuppression in the effector phase. Delivery of an intrathecal IFN-γ expression system during the initiation phase triggered an earlier disease onset followed by recovery, while overexpression of IFN-γ in the chronic phase resulted in disease amelioration (37). Consistently, intraventricular injection of IFN-γ during the initiation phase in CREAE mice increased the number of relapses (38). More recently, Naves et al. showed that IFNGR-deficient mice exhibited delayed disease onset followed by a more severe chronic phase, compared to WT mice (31). Similar results have been found analyzing mice lacking the IFN-γ gene or injecting an anti-IFN-γ neutralizing antibody during EAE progression (39). Furthermore, the administration of IFN-γ to EAE mice during the inductive period led to disease exacerbation, while such treatment was protective during the effector phase (31). Interestingly, the immunosuppressive activity of IFN-γ required functional type I IFN signaling and signal transducer and activator of transcription (STAT)-1 (31). In this way, stage-specific functions of IFN-γ can reconcile previous conflicting results in EAE and might also explain the mixed outcome reported in RRMS patients treated with IFN-γ (19).

IFN-γ and Immune Cells

Compelling evidence shows that IFN-γ exerts opposing effects on immune cells during the development of EAE and MS. In addition, several innate and adaptive immune cells play a dual role during the progression of these diseases associated with their IFN-γ production (**Figure 1**). Below, we will review and discuss this literature, focusing on the less-known protective face of IFN-γ (**Table 1**).

Innate Immune Cells

Macrophages and Neutrophils

IFN-γ controls the infiltration of macrophages and neutrophils into the CNS regulating the course of EAE (74). Animals deficient in IFN-γ or IFNGR generate an atypical disease affecting mainly the brainstem and cerebellum with increased expression of CXCL2, favoring the recruitment of high numbers of CXCR2-mediated neutrophils; while in conventional EAE, IFN-γ leads to

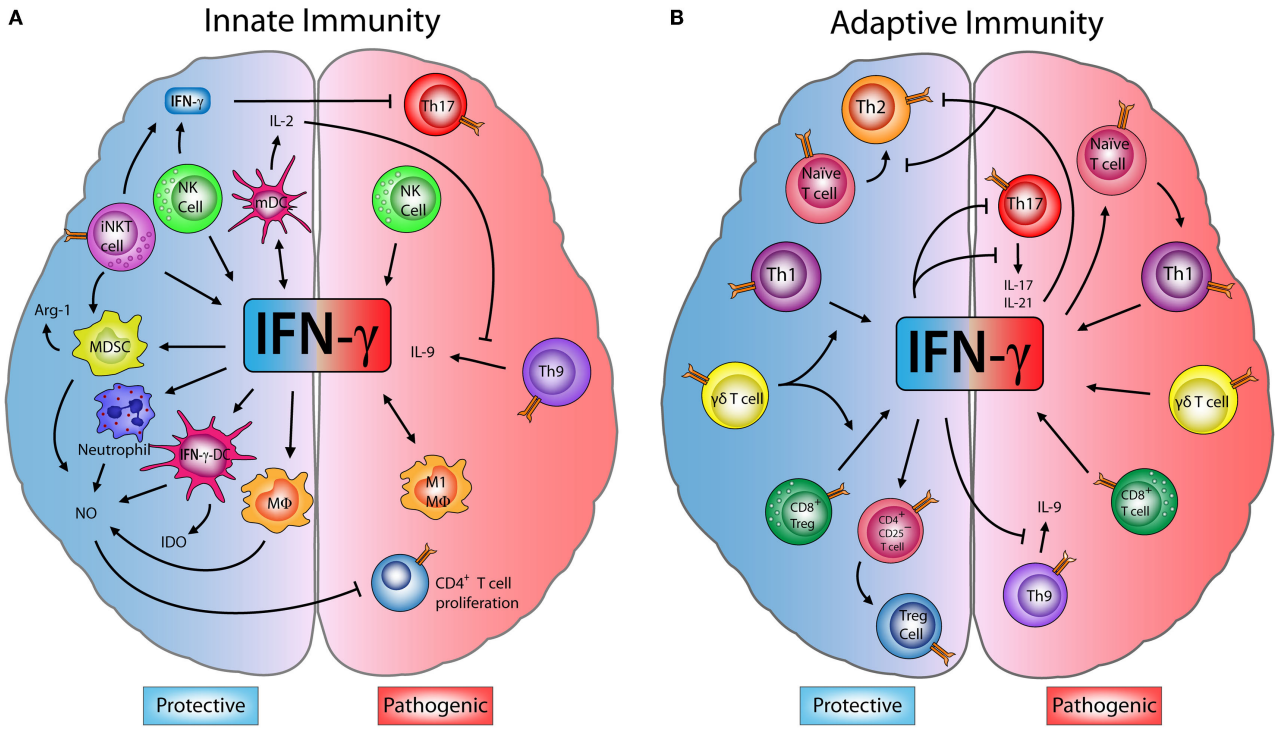

FIGURE 1 | Dual role of IFN-γ in innate and adaptive immune cells in EAE. **(A)** *Innate immunity*: M1-macrophages (M1-MΦ) and natural killer (NK) cells produce interferon (IFN)-γ, which has a pathogenic role exacerbating encephalomyelitis autoimmune experimental (EAE) symptoms. However, some studies have shown that IFN-γ produced by NK and invariant NKT (iNKT) cells inhibits effector Th17 cells, decreasing the disease severity. IFN-γ induces the production of nitric oxide (NO) in neutrophils, MΦ, myeloid-derived suppressor cells (MDSCs), and IFN-γ-induced dendritic cells (IFN-γ-DC). NO can directly inhibit the proliferation of $CD4^+$ T cells. IFN-γ also induces the expression of indoleamine 2,3-dioxygenase (IDO) in IFN-γ-DC and arginase-1 (Arg-1) by MDSC, enzymes that can suppress inflammation. Furthermore, IFN-γ induces IL-27 production by mature dendritic cells (mDC) which blocks Th9 differentiation and IL-9 production, controlling disease progression. **(B)** *Adaptive immunity*: IFN-γ secreted by Th1 and $CD8^+$ T cells has an inflammatory effect and can drive the onset and progression of EAE. Despite this, IFN-γ is able to block Th9 cells, while Th1-secreted IFN-γ inhibits Th2 and Th17 effector cells. Interestingly, IFN-γ can induce $CD4^+CD25^+$ regulatory T cells (Tregs) increasing their FOXP3 expression. Upon transfer, these IFN-γ-induced Tregs limit the severity of EAE. Moreover, IFN-γ production by $CD8^+$ regulatory T cells ($CD8^+$ Tregs) also reduces EAE symptoms. Finally, IFN-γ produced by γδ T cells worsens EAE, but has a regulatory role on the production of IFN-γ by T cells, which is necessary to limit disease.

increased CCL2 levels guiding macrophage infiltration into the spinal cord mediated by CCR2 (40–42). Macrophages and neutrophils produce high levels of nitric oxide (NO), which has both pathogenic and regulatory functions in neuroinflammation (75). Interestingly, IFN-γ is a primary inducer of NO and mice deficient in inducible nitric oxide synthase (iNOS) develop a severe form of EAE (76, 77). Willenborg et al. showed that peritoneal exudate cells (PEC), characterized by a high presence of macrophages, are able to inhibit the extensive proliferation of splenocytes from IFNGR-deficient mice with EAE by IFN-γ-dependent NO production (33). Neutrophils and myeloid-derived suppressor cells (MDSCs) with high expression of Gr-1 also exhibited potent suppressor activity in EAE, inhibiting T cell proliferation through a mechanism that was absolutely dependent on IFN-γ and NO (43–45). Additionally, IFN-γ along with interleukin (IL)-4, secreted by activated invariant NKT (iNKT) cells, stimulated MDSCs to suppress EAE via iNOS and arginase (arg)-1 expression (51).

Natural Killer Cells

NK cells play both a regulatory and pathogenic role in EAE and MS (39, 46, 78–86). Although the underlying mechanisms are poorly understood, several studies suggest that IFN-γ-producing NK cells might be driving this duality in a location and stage-dependent manner (39, 46–49, 87). NK cells have been identified as the main source of IFN-γ production in the initiation stage of EAE, which might be necessary for migration of pathogenic T cells into the CNS (39). Interestingly, early but not late depletion of NK cells significantly delayed the onset of disease (39). IL-18 and IL-21 are two key cytokines involved in NK cell functional maturation (88, 89). Administration of IL-21 before EAE immunization promoted higher IFN-γ production by NK cells and induced a significantly enhanced acute phase with more intense CNS cell infiltration compared to untreated mice (90). However, IL-21 treatment failed to induce augmentation of IFN-γ production and had no effect on disease progression when applied for one week starting a few days before disease onset (90). Similarly, IL-18 injection in WT mice at the time of immunization enhanced disease severity promoting autoreactive Th1 cell development through the induction of IFN-γ by NK cells (87). Moreover, IFN-γ signaling in NK cells was required to restore EAE susceptibility in IL-18 defective mice (87). Taken together, these results suggest that early IFN-γ production by NK cells mainly contributes to the

TABLE 1 | The protective effects of IFN-γ and IFN-γ-producing immune cells in EAE and MS.

Cell type	Experimental design	Effects of IFN-γ	Reference
Macrophages	*In vitro* culture of IFNGR-deficient PEC	IFN-γ induces PEC NO-expression inhibiting proliferation of splenocytes	(33)
Neutrophils	Induced EAE in IFN-γ and IFNGR-deficient mice	IFN-γ restricts neutrophils infiltration in the brainstem and cerebellum primarily by regulating CXCL2 expression	(40–42)
	In vitro analysis of $Gr1^+$ neutrophils sorted from CNS of mice with EAE	IFN-γ secreted by T cells induced NO production by $Gr1^+$ neutrophils which inhibited T cell proliferation	(43)
Myeloid-derived suppressor cells (MDSCs)	Analysis of $CD11b^+$ $Gr1^+$ MDSC from EAE mice	IFN-γ secreted by activated T cells induced MDSC inhibiting $CD4^+$ T cells proliferation by NO-dependent manner	(44)
	EAE mice treated with anti-IFN-γ	Anti-IFN-γ reduced MDSCs frequency and increased EAE severity	(45)
Natural killer cells (NK)	EAE mice treated with anti-IFN-γ	Decreased Th17-characteristic transcription factors expression due to modulation of microglia activation	(46)
	HINT1/Hsp70 protein complex from brains of PLP-sensitized SJL/J mice injected into congenic mice before immunization	Upregulated MHC class I peptide H60 expression, increased NK cell IFN-γ production, inhibited IL-17 production, and prevented EAE	(47–49)
	Analysis of NK cell functionality in human PBMC	RRMS patients exhibit impaired response to IL-12 and severely diminished IFN-γ production in $CD3^-CD56^{bright}CD16^-$ NK cells	(50)
Invariant NKT cells	*In vivo* IFN-γ neutralization in αGalCer-treated mice with EAE. *In vitro* iNKT analysis	Increased production of IFN-γ, IL-4, and IL-10 by iNKT cells which mediated the suppression of Th17 cells and increased EAE regulation by MDSCs	(51–53)
Dendritic cells (DC)	Transfer of IFN-γ treated DC into murine EAE models	Induced an incompletely mature DC phenotype and decreased disease severity and relapse frequency	(54)
	In vitro analysis of splenocytes isolated from WT and IFN-γ-deficient EAE mice	Induced DC IL-27 expression which inhibited Th9 cell differentiation and IL-9 production by Th9 and Th17 cells	(55)
$CD4^+$ T lymphocytes	IFN-γ added to CD3-activated PBMC from chronic-progressive MS patients	Lymphocyte proliferation inhibition in an IFN-γ dose-dependent manner	(56)
	Analysis of IFN-γ deficient mice with EAE	Increased apoptosis and inhibited proliferation *in vivo* and *ex vivo* of $CD4^+CD44^{high}$ T cells in spleen and CNS	(57)
	Study of IFN-γ and IFNGR EAE deficient mice	Inhibited Th17 differentiation and IL-17 production	(31, 58–62)
	IFN-γ deficient EAE mice treated with anti-IL-9	Decreased Th9 differentiation and IL-9 production *in vitro* and *in vivo* in the CNS of mice with EAE	(55)
	$CD4^+$ T cells transfected with IFN-γ expressing vector transferred into EAE mice	Th1 IFN-$\gamma^{high}CD25^-FOXP3^-$ suppresses Th17 effector cells and decreased EAE severity	(63)
γδ T cells	EAE generated in bone marrow chimera with γδ and IFN-γ-deficient mice	γδ T cells promotes the expression of IFN-γ by T cells with a reduction of EAE severity	(64)
$CD4^+$ Tregs	*In vitro* addition of IFN-γ to mice and human $CD4^+CD25^-$ T cell cultures. IFN-γ-converted Tregs injected into EAE mice	IFN-γ-converted Tregs inhibited T cell proliferation in mice and human cells. Administration of these cells ameliorated EAE severity	(65)
$CD8^+$ T lymphocytes	Transfer of MOG-induced $CD8^+$ T cells from IFN-γ-deficient mice into wild-type mice before EAE induction	Amelioration of EAE severity mediated by $CD8^+$ T cell IFN-γ production	(66)
	Analysis of $CD8^+LAP^+$ T cells from IFN-γ and IFNGR-deficient mice and transfer into EAE	IFN-γ production by $CD8^+LAP^+$ T cells inhibited T cell proliferation and reduced severity of EAE.	(67)
	Vaccination with a TCR-derived peptide before EAE induction in WT and IFN-γ KO mice	Vaccination activates $CD8\alpha\alpha^+TCR\alpha\beta^+$ T cells and delayed EAE onset in an IFN-γ mediated fashion	(68–70)
	Isolation of human and mice $CD8^+CD38^{high}$ T cells. *In vivo* injection of $CD8^+CD38^{high}$ into EAE mice	IFN-γ production by $CD8^+CD38^{high}$ T cells inhibit T cell proliferation in human and mice. These cells decreased disease severity and delayed onset of EAE.	(71)
	MS patients and EAE mice treated with Glatiramer acetate (GA)	GA increases $CD8^+$ T proliferation and IFN-γ levels in MS and IDO and IFN-γ-producing $CD8^+$ T cells in EAE	(72, 73)
B cells	IFN-γ treatment in early EAE stage in marmoset	Reduced plasma MOG-specific IgG levels	(35)

initiation, but not progression, of EAE pathogenesis. By contrast, once NK cells infiltrate the CNS, they assume a protective role suppressing myelin-reactive Th17 cells via modulation of microglia activation. This effect was CNS compartment-restricted and was perforin and IFN-γ-dependent (46). Therefore, signals and/or components generated in the CNS during the effector phase might be inducing protective functions in infiltrating NK cells. Indeed, peptides complexed with the chaperone heat shock protein (Hsp) 70 derived from inflamed brain of EAE mice have been described as promotors of the immunotolerogenic activity

of NK cells in EAE. Suppressive effects of Hsp-peptide complex-activated NK cells correlated with high production of IFN-γ and resulted in inhibition of Th17 cells (47–49).

NK cell subtypes and iNKT cells may also have IFN-γ-mediated suppressive activity in MS and EAE. Regarding MS, evidence in two independent cohorts of RRMS patients demonstrated that the classically inhibitory $CD3^{-}CD56^{bright}CD16^{-}$ NK cells from RRMS patients have impaired expansion in response to IL-12, and severely diminished IFN-γ production compared to healthy control NK cells (50). In addition, *in vivo* activation of iNKT cells at the same time as EAE induction significantly ameliorated disease progression through mechanisms dependent on IFN-γ alone (52) or synergistically with IL-4 and IL-10, resulting in inhibition of the Th17 response (53).

Dendritic Cells

Dendritic cells (DC) are professional APC important to maintain the balance between immunity and tolerance. In EAE, they efficiently present myelin antigens in order to prime and polarize naïve T cells. They also help regulate EAE severity as evidenced by disease exacerbation in DC deficient mice (91, 92). The regulatory effects of DC are partly due to the IFN-γ-induced production of IL-27 that suppressed the differentiation and encephalitogenicity of Th9 cells. It also inhibited the production of IL-9 by both Th9 and Th17 cells. This suppression was partially dependent on STAT-1 and T-bet and was necessary to regulate EAE severity (55). Remarkably, splenic DC exposed to IFN-γ for 48 hours exhibited an immature and tolerogenic phenotype (tol-DC). These tol-DC decreased disease severity in Lewis rats and relapse frequency in SJL/J and B6 mouse models when transferred during the inductive phase (54). EAE amelioration was accompanied by reduced macrophage activation and $CD4^{+}$ T cell CNS infiltration, compared to control mice. The therapeutic activity was dependent on an antigen-specific IFN-γ pathway, involving increased DC expression of indoleamine 2,3-dioxygenase (IDO), which induced $CD4^{+}$ T cell apoptosis (54).

Adaptive Immune Cells

$CD4^{+}$ T Lymphocytes

$CD4^{+}$ T (Th) cells proliferate and differentiate into various subtypes in response to antigen stimulation and their microenvironment in order to exert specific effector or regulatory functions (93). Effector $CD4^{+}$ T (Teff) cell lineages, such as Th1 cells, Th2 cells, Th17 cells, and Th9 cells, and regulatory T cells (Tregs) can be distinguished by the cytokines they produce and the transcription factors essential for their differentiation. These T cells also exhibit functional and phenotypical plasticity expressing cytokines and/or transcription factors of other lineages (94, 95).

Classically, IFN-γ is known for promoting the differentiation of Th1 cells and inhibiting the Th2 immune response which may contribute to neuroinflammation (5, 10, 96, 97). Despite its inflammatory activity, IFN-γ increased apoptosis and inhibited proliferation of $CD4^{+}CD44^{high}$ (activated) T lymphocytes from both the spleen and CNS of EAE mice (57). Notably, it also inhibited *in vitro* proliferation of T cell receptor (TCR)-activated peripheral blood mononuclear cells (PBMC) from progressive MS patients in a dose-dependent manner (56). Mice depleted of IFN-γ or IFN-γ signaling developed more severe EAE, atypical neurological symptoms, and increased Th17-characteristic inflammation. These data underscore an important anti-inflammatory function of IFN-γ in EAE: the inhibition of pathogenic Th17 cell differentiation and cytokine production (5, 31, 58–62, 96). Besides, it has been shown that IFN-γ has a STAT-1-mediated direct inhibitory effect on pathogenic Th9 cells (55). Interestingly, another study identified a non-pathogenic Th1 cell subset with high IFN-γ expression, capable of restraining EAE development during early stages of disease by suppressing Th17 cells in an IFN-γ-dependent manner (63). The inhibitory mechanism involved the activation of STAT-1 and IL-21 expression via induction of T-bet (60, 62). Despite the ability of IFN-γ to directly and indirectly inhibit Th17 cells, a pathogenic population of Th1 cells has been identified in EAE and MS that also expresses IL-17. This capacity to express both cytokines (IFN-γ and IL-17) may be due to the plasticity of Th17 cells, which can undergo a shift toward the Th1 phenotype (95, 98, 99).

γδ T Cells

Several studies have shown that γδ T cells are present in the CNS of MS patients and EAE mice (100). Given that activated γδ T cells have the capacity to produce high expression of Th1 and Th17 cytokines, they might contribute to the induction or maintenance of neuroinflammation. However, efforts to determine a role for these cells have given contradictory results. While some studies have found that depletion of γδ T cells resulted in reduced severity of EAE, other reports have described disease aggravation (100). Regarding IFN-γ, evidence suggests that during early EAE, γδ T cells may act either as a main source of this cytokine (101) or regulate IFN-γ expression in other cell types, including $CD4^{+}$ and $CD8^{+}$ T cells (64). Indeed, SJL/J mice depleted of γδ T cells showed a significant reduction of IFN-γ expression in the CNS at all stages of EAE (102). Other studies have shown that mice deficient in γδ T cells that are reconstituted with γδ T cells lacking IFN-γ expression developed a significantly delayed and attenuated EAE. This suggested that IFN-γ production by γδ T cells may be central to initial inflammatory events (101). Despite this, Ponomarev et al. proposed that γδ T cells are required to promote CNS-restricted production of sufficient levels of IFN-γ necessary for EAE recovery (64).

$CD4^{+}$ Regulatory T Lymphocytes

It has been reported that IFN-γ is important to the function of Tregs in EAE and MS. Reduced FoxP3 expression and lower frequency and function of Tregs was reported in IFN-γ-deficient mice with EAE, in comparison to EAE-induced WT mice (65). Remarkably, treatment of $CD4^{+}CD25^{-}$ T cells from WT or IFN-γ-deficient mice with IFN-γ alone or with additional TCR stimulation led to their conversion into Tregs expressing CD25 and FOXP3 (65). These IFN-γ-induced Tregs effectively inhibited EAE disease progression when adoptively transferred into IFN-γ-deficient mice. Human $CD4^{+}CD25^{-}$ T cells from healthy volunteers were similarly converted into functionally active Tregs *ex vivo* upon IFN-γ stimulation (65).

A new subpopulation of Tregs expressing T-bet, CXCR3 and IFN-γ, named Th1-like Tregs has been reported in healthy individuals (103) and have regulatory functions focused on

Th1-mediated inflammatory diseases (104–108). Interestingly, these cells were also described in MS and EAE (109, 110). An increased frequency of Th1-like Tregs with reduced suppressive function was reported in untreated RRMS patients compared to healthy controls (110). In this case, addition of IFN-γ neutralizing antibodies recovered their functionality suggesting that IFN-γ might contribute to their reduced immunomodulatory capacity (110).

CD8$^+$ T Lymphocytes

Several studies have demonstrated that IFN-γ production by CD8$^+$ T cells is a major mediator of EAE induced by cytotoxic T lymphocytes (CTL) (111–114). One of these investigations showed that atypical EAE induced by intrathecal transfer of myelin basic protein (MBP)-specific CD8$^+$ T cells in C3H mice was ameliorated by co-injection with neutralizing antibodies for IFN-γ (112). Other studies have identified subsets of regulatory CD8$^+$ T cells (CD8$^+$ Tregs) that suppress EAE development via IFN-γ-dependent mechanisms. Both therapeutic and prophylactic transfer of myelin oligodendroctye glycoprotein MOG-induced CD8$^+$ T cells into mice with EAE ameliorated disease suppressing the chronic phase, but not affecting the disease onset or acute phase (66, 115). Strikingly, this protective function was lost when IFN-γ-deficient MOG-induced-CD8$^+$ T cells were transferred before EAE induction in WT mice, but was enhanced when IFN-γ production was stimulated in MOG-specific CD8$^+$ T cells before cell transfer (66). Furthermore, in those studies reporting a pathogenic function, myelin-specific CD8$^+$ T cell lines used to passively induce EAE were generated from CD8$^+$ T cells isolated during the inductive phase (111, 112). In contrast, regulatory myelin-specific CD8$^+$ T cells were obtained during the chronic phase of disease (66, 115). Taken together, these results reinforce the notion of a stage-specific IFN-γ-dependent regulation, mediated in this case by CNS-specific regulatory CD8$^+$ T cells.

A naturally occurring CD8$^+$ Tregs subset was identified that expressed latency-associated peptide (LAP) on their cell surface and produced more IFN-γ than their LAP$^-$ counterparts. Adoptive transfer of these cells previous to myelin immunization improved EAE recovery mediated by their IFN-γ production (67). A CD8αα$^+$TCRαβ$^+$ T cell subset capable of preventing EAE when stimulated with a TCR-derived peptide before MBP-peptide immunization in H-2u mouse strains has also been described (68, 69, 116). Interestingly, the vaccine failed to prevent EAE development in IFN-γ-deficient mice and resulted in delayed disease onset but worsened disease severity compared to control mice, suggesting an important stage-specific role for IFN-γ signaling in CD8αα$^+$TCRαβ$^+$ T cell-mediated protection (70, 116). A CD8$^+$ Tregs subtype expressing high levels of CD38 ectonucleotidase suppressed Teff cell proliferation in a non-antigen specific, cell-to-cell contact, and IFN-γ-dependent fashion, resulting in ameliorated EAE (71). Finally, IFN-γ-producing CD8$^+$ T cells induced by glatiramer acetate (GA), a therapy for MS, suppressed EAE in mice via an IDO-dependent mechanism, suggesting that the immunomodulatory action of GA is mediated at least in part by IFN-γ production by CD8$^+$ T cells (72). Consistently, GA-specific CD8$^+$ T cells from GA-treated RRMS patients tended to produce more IFN-γ than CD8$^+$ T cells from untreated patients (73). In progressive MS patients, CTL had impaired IL-2 induced IFN-γ production and decreased ability to suppress proliferation of TCR-stimulated autologous lymphocytes (56).

B Lymphocytes

The effect of IFN-γ on B cells in the neuroinflammatory context of MS and EAE is unclear. Bar-Or and colleagues demonstrated that CD19$^+$ B cells isolated from RRMS patients had significantly increased production of lymphotoxin (TNF-β) and TNF-α in response to IFN-γ and insignificant changes in IL-10 production (117). In marmoset EAE, exogenous administration of IFN-γ caused no significant clinical change in disease; however, there was a significant decrease in plasma IgG specific to MOG peptides (35).

Concluding Remarks

Recent studies support the notion that IFN-γ exerts a stage-specific role during EAE development. Strikingly, several innate and adaptive immune cells develop opposite activities during EAE progression, which is related to their production of IFN-γ in a stage-specific manner. Furthermore, the suppressive activity of different types of immune regulatory cells is IFN-γ-dependent. Taken together, these data provide a mechanistic basis explaining the previous controversial results in relation to the role of IFN-γ in EAE and MS. Delineating the varying activities of IFN-γ as well as the role of IFN-γ-producing immune cells during the course of EAE and MS will not only provide insight into the complex role of IFN-γ in these diseases but might also lead to therapies targeting IFN-γ signaling or IFN-γ-producing immune cells. These treatments can be helpful to a selective group of MS patients or during a specific stage of disease.

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References

1. Borden EC, Sen GC, Uze G, Silverman RH, Ransohoff RM, Foster GR, et al. Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov* (2007) **6**(12):975–90. doi:10.1038/nrd2422
2. Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. *Annu Rev Immunol* (1993) **11**:571–611. doi:10.1146/annurev.iy.11.040193.003035
3. Shachar I, Karin N. The dual roles of inflammatory cytokines and chemokines in the regulation of autoimmune diseases and their clinical implications. *J Leukoc Biol* (2013) **93**(1):51–61. doi:10.1189/jlb.0612293
4. Kelchtermans H, Billiau A, Matthys P. How interferon-gamma keeps autoimmune diseases in check. *Trends Immunol* (2008) **29**(10):479–86. doi:10.1016/j.it.2008.07.002
5. Nylander A, Hafler DA. Multiple sclerosis. *J Clin Invest* (2012) **122**(4):1180–8. doi:10.1172/JCI58649
6. Mahad DH, Trapp BD, Lassmann H. Pathological mechanisms in progressive multiple sclerosis. *Lancet Neurol* (2015) **14**(2):183–93. doi:10.1016/S1474-4422(14)70256-X
7. Steinman L, Zamvil SS. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. *Ann Neurol* (2006) **60**(1):12–21. doi:10.1002/ana.20913

8. Baxter AG. The origin and application of experimental autoimmune encephalomyelitis. *Nat Rev Immunol* (2007) **7**(11):904–12. doi:10.1038/nri2190
9. Kurschus FC. T cell mediated pathogenesis in EAE: molecular mechanisms. *Biomed J* (2015) **38**(3):183–93. doi:10.4103/2319-4170.155590
10. Lara Sanvito, Constantinescu CS, Bruno Gran, 't Hart Bert A. The multifaceted role of interferon-γ central nervous system autoimmune demyelination. *Open Autoimmunity J* (2010) **2**:151–9.
11. Olsson T. Cytokines in neuroinflammatory disease: role of myelin autoreactive T cell production of interferon-gamma. *J Neuroimmunol* (1992) **40**(2–3):211–8. doi:10.1016/0165-5728(92)90135-8
12. Brosnan CF, Cannella B, Battistini L, Raine CS. Cytokine localization in multiple sclerosis lesions: correlation with adhesion molecule expression and reactive nitrogen species. *Neurology* (1995) **45**(6 Suppl 6):S16–21. doi:10.1212/WNL.45.6_Suppl_6.S16
13. Renno T, Krakowski M, Piccirillo C, Lin JY, Owens T. TNF-alpha expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis. Regulation by Th1 cytokines. *J Immunol* (1995) **154**(2):944–53.
14. Renno T, Lin JY, Piccirillo C, Antel J, Owens T. Cytokine production by cells in cerebrospinal fluid during experimental allergic encephalomyelitis in SJL/J mice. *J Neuroimmunol* (1994) **49**(1–2):1–7. doi:10.1016/0165-5728(94)90174-0
15. Merrill JE, Kono DH, Clayton J, Ando DG, Hinton DR, Hofman FM. Inflammatory leukocytes and cytokines in the peptide-induced disease of experimental allergic encephalomyelitis in SJL and B10.PL mice. *Proc Natl Acad Sci USA* (1992) **89**(2):574–8. doi:10.1073/pnas.89.2.574
16. Pettinelli CB, McFarlin DE. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes. *J Immunol* (1981) **127**(4):1420–3.
17. Bettelli E, Sullivan B, Szabo SJ, Sobel RA, Glimcher LH, Kuchroo VK. Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp Med* (2004) **200**(1):79–87. doi:10.1084/jem.20031819
18. Nath N, Prasad R, Giri S, Singh AK, Singh I. T-bet is essential for the progression of experimental autoimmune encephalomyelitis. *Immunology* (2006) **118**(3):384–91. doi:10.1111/j.1365-2567.2006.02385.x
19. Panitch HS, Hirsch RL, Schindler J, Johnson KP. Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system. *Neurology* (1987) **37**(7):1097–102. doi:10.1212/WNL.37.7.1097
20. Skurkovich S, Boiko A, Beliaeva I, Buglak A, Alekseeva T, Smirnova N, et al. Randomized study of antibodies to IFN-gamma and TNF-alpha in secondary progressive multiple sclerosis. *Mult Scler* (2001) **7**(5):277–84. doi:10.1191/135245801681137960
21. Billiau A, Heremans H, Vandekerckhove F, Dijkmans R, Sobis H, Meulepas E, et al. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J Immunol* (1988) **140**(5):1506–10.
22. Voorthuis JA, Uitdehaag BM, De Groot CJ, Goede PH, van der Meide PH, Dijkstra CD. Suppression of experimental allergic encephalomyelitis by intraventricular administration of interferon-gamma in Lewis rats. *Clin Exp Immunol* (1990) **81**(2):183–8. doi:10.1111/j.1365-2249.1990.tb03315.x
23. Heremans H, Dillen C, Groenen M, Martens E, Billiau A. Chronic relapsing experimental autoimmune encephalomyelitis (CREAE) in mice: enhancement by monoclonal antibodies against interferon-gamma. *Eur J Immunol* (1996) **26**(10):2393–8. doi:10.1002/eji.1830261019
24. Duong TT, St Louis J, Gilbert JJ, Finkelman FD, Strejan GH. Effect of anti-interferon-gamma and anti-interleukin-2 monoclonal antibody treatment on the development of actively and passively induced experimental allergic encephalomyelitis in the SJL/J mouse. *J Neuroimmunol* (1992) **36**(2–3):105–15. doi:10.1016/0165-5728(92)90042-J
25. Lublin FD, Knobler RL, Kalman B, Goldhaber M, Marini J, Perrault M, et al. Monoclonal anti-gamma interferon antibodies enhance experimental allergic encephalomyelitis. *Autoimmunity* (1993) **16**(4):267–74. doi:10.3109/08916939309014645
26. Duong TT, Finkelman FD, Singh B, Strejan GH. Effect of anti-interferon-gamma monoclonal antibody treatment on the development of experimental allergic encephalomyelitis in resistant mouse strains. *J Neuroimmunol* (1994) **53**(1):101–7. doi:10.1016/0165-5728(94)90069-8
27. Krakowski M, Owens T. Interferon-gamma confers resistance to experimental allergic encephalomyelitis. *Eur J Immunol* (1996) **26**(7):1641–6. doi:10.1002/eji.1830260735
28. Ferber IA, Brocke S, Taylor-Edwards C, Ridgway W, Dinisco C, Steinman L, et al. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol* (1996) **156**(1):5–7.
29. Sabatino JJ Jr, Shires J, Altman JD, Ford ML, Evavold BD. Loss of IFN-gamma enables the expansion of autoreactive CD4+ T cells to induce experimental autoimmune encephalomyelitis by a nonencephalitogenic myelin variant antigen. *J Immunol* (2008) **180**(7):4451–7. doi:10.4049/jimmunol.180.7.4451
30. Willenborg DO, Fordham S, Bernard CC, Cowden WB, Ramshaw IA. IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *J Immunol* (1996) **157**(8):3223–7.
31. Naves R, Singh SP, Cashman KS, Rowse AL, Axtell RC, Steinman L, et al. The interdependent, overlapping, and differential roles of type I and II IFNs in the pathogenesis of experimental autoimmune encephalomyelitis. *J Immunol* (2013) **191**(6):2967–77. doi:10.4049/jimmunol.1300419
32. Xiao BG, Ma CG, Xu LY, Link H, Lu CZ. IL-12/IFN-gamma/NO axis plays critical role in development of Th1-mediated experimental autoimmune encephalomyelitis. *Mol Immunol* (2008) **45**(4):1191–6. doi:10.1016/j.molimm.2007.07.003
33. Willenborg DO, Fordham SA, Staykova MA, Ramshaw IA, Cowden WB. IFN-gamma is critical to the control of murine autoimmune encephalomyelitis and regulates both in the periphery and in the target tissue: a possible role for nitric oxide. *J Immunol* (1999) **163**(10):5278–86.
34. Wheeler RD, Zehntner SP, Kelly LM, Bourbonniere L, Owens T. Elevated interferon gamma expression in the central nervous system of tumour necrosis factor receptor 1-deficient mice with experimental autoimmune encephalomyelitis. *Immunology* (2006) **118**(4):527–38. doi:10.1111/j.1365-2567.2006.02395.x
35. Jagessar SA, Gran B, Heijmans N, Bauer J, Laman JD, 't Hart BA, et al. Discrepant effects of human interferon-gamma on clinical and immunological disease parameters in a novel marmoset model for multiple sclerosis. *J Neuroimmune Pharmacol* (2012) **7**(1):253–65. doi:10.1007/s11481-012-9384-x
36. Bever CT Jr, Panitch HS, Levy HB, McFarlin DE, Johnson KP. Gamma-interferon induction in patients with chronic progressive MS. *Neurology* (1991) **41**(7):1124–7. doi:10.1212/WNL.41.7.1124
37. Furlan R, Brambilla E, Ruffini F, Poliani PL, Bergami A, Marconi PC, et al. Intrathecal delivery of IFN-gamma protects C57BL/6 mice from chronic-progressive experimental autoimmune encephalomyelitis by increasing apoptosis of central nervous system-infiltrating lymphocytes. *J Immunol* (2001) **167**(3):1821–9. doi:10.4049/jimmunol.167.3.1821
38. Tanuma N, Shin T, Kogure K, Matsumoto Y. Differential role of TNF-alpha and IFN-gamma in the brain of rats with chronic relapsing autoimmune encephalomyelitis. *J Neuroimmunol* (1999) **96**(1):73–9. doi:10.1016/S0165-5728(99)00018-1
39. Dungan LS, McGuinness NC, Boon L, Lynch MA, Mills KH. Innate IFN-gamma promotes development of experimental autoimmune encephalomyelitis: a role for NK cells and M1 macrophages. *Eur J Immunol* (2014) **44**(10):2903–17. doi:10.1002/eji.201444612
40. Tran EH, Prince EN, Owens T. IFN-gamma shapes immune invasion of the central nervous system via regulation of chemokines. *J Immunol* (2000) **164**(5):2759–68. doi:10.4049/jimmunol.164.5.2759
41. Lees JR, Golumbek PT, Sim J, Dorsey D, Russell JH. Regional CNS responses to IFN-gamma determine lesion localization patterns during EAE pathogenesis. *J Exp Med* (2008) **205**(11):2633–42. doi:10.1084/jem.20080155
42. Stoolman JS, Duncker PC, Huber AK, Segal BM. Site-specific chemokine expression regulates central nervous system inflammation and determines clinical phenotype in autoimmune encephalomyelitis. *J Immunol* (2014) **193**(2):564–70. doi:10.4049/jimmunol.1400825
43. Zehntner SP, Brickman C, Bourbonniere L, Remington L, Caruso M, Owens T. Neutrophils that infiltrate the central nervous system regulate T cell responses. *J Immunol* (2005) **174**(8):5124–31. doi:10.4049/jimmunol.174.8.5124

44. Zhu B, Bando Y, Xiao S, Yang K, Anderson AC, Kuchroo VK, et al. CD11b+*Ly-6C*(hi) suppressive monocytes in experimental autoimmune encephalomyelitis. *J Immunol* (2007) **179**(8):5228–37. doi:10.4049/jimmunol.179.8.5228
45. Pastor S, Minguela A, Mi W, Ward ES. Autoantigen immunization at different sites reveals a role for anti-inflammatory effects of IFN-gamma in regulating susceptibility to experimental autoimmune encephalomyelitis. *J Immunol* (2009) **182**(9):5268–75. doi:10.4049/jimmunol.0800681
46. Hao J, Liu R, Piao W, Zhou Q, Vollmer TL, Campagnolo DI, et al. Central nervous system (CNS)-resident natural killer cells suppress Th17 responses and CNS autoimmune pathology. *J Exp Med* (2010) **207**(9):1907–21. doi:10.1084/jem.20092749
47. Galazka G, Jurewicz A, Domowicz M, Cannella B, Raine CS, Selmaj K. HINT1 peptide/Hsp70 complex induces NK-cell-dependent immunoregulation in a model of autoimmune demyelination. *Eur J Immunol* (2014) **44**(10):3026–44. doi:10.1002/eji.201444694
48. Galazka G, Jurewicz A, Orlowski W, Stasiolek M, Brosnan CF, Raine CS, et al. EAE tolerance induction with Hsp70-peptide complexes depends on H60 and NKG2D activity. *J Immunol* (2007) **179**(7):4503–12. doi:10.4049/jimmunol.179.7.4503
49. Galazka G, Stasiolek M, Walczak A, Jurewicz A, Zylicz A, Brosnan CF, et al. Brain-derived heat shock protein 70-peptide complexes induce NK cell-dependent tolerance to experimental autoimmune encephalomyelitis. *J Immunol* (2006) **176**(3):1588–99. doi:10.4049/jimmunol.176.3.1588
50. Lunemann A, Tackenberg B, DeAngelis T, da Silva RB, Messmer B, Vanoaica LD, et al. Impaired IFN-gamma production and proliferation of NK cells in multiple sclerosis. *Int Immunol* (2011) **23**(2):139–48. doi:10.1093/intimm/dxq463
51. Parekh VV, Wu L, Olivares-Villagomez D, Wilson KT, Van Kaer L. Activated invariant NKT cells control central nervous system autoimmunity in a mechanism that involves myeloid-derived suppressor cells. *J Immunol* (2013) **190**(5):1948–60. doi:10.4049/jimmunol.1201718
52. Furlan R, Bergami A, Cantarella D, Brambilla E, Taniguchi M, Dellabona P, et al. Activation of invariant NKT cells by alphaGalCer administration protects mice from MOG35-55-induced EAE: critical roles for administration route and IFN-gamma. *Eur J Immunol* (2003) **33**(7):1830–8. doi:10.1002/eji.200323885
53. Mars LT, Araujo L, Kerschen P, Diem S, Bourgeois E, Van LP, et al. Invariant NKT cells inhibit development of the Th17 lineage. *Proc Natl Acad Sci USA* (2009) **106**(15):6238–43. doi:10.1073/pnas.0809317106
54. Xiao BG, Wu XC, Yang JS, Xu LY, Liu X, Huang YM, et al. Therapeutic potential of IFN-gamma-modified dendritic cells in acute and chronic experimental allergic encephalomyelitis. *Int Immunol* (2004) **16**(1):13–22. doi:10.1093/intimm/dxh003
55. Murugaiyan G, Beynon V, Pires Da Cunha A, Joller N, Weiner HL. IFN-gamma limits Th9-mediated autoimmune inflammation through dendritic cell modulation of IL-27. *J Immunol* (2012) **189**(11):5277–83. doi:10.4049/jimmunol.1200808
56. Balashov KE, Khoury SJ, Hafler DA, Weiner HL. Inhibition of T cell responses by activated human CD8+ T cells is mediated by interferon-gamma and is defective in chronic progressive multiple sclerosis. *J Clin Invest* (1995) **95**(6):2711–9. doi:10.1172/JCI117973
57. Chu CQ, Wittmer S, Dalton DK. Failure to suppress the expansion of the activated CD4 T cell population in interferon gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. *J Exp Med* (2000) **192**(1):123–8. doi:10.1084/jem.192.1.123
58. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* (2005) **6**(11):1133–41. doi:10.1038/ni1261
59. Berghmans N, Nuyts A, Uyttenhove C, Van Snick J, Opdenakker G, Heremans H. Interferon-gamma orchestrates the number and function of Th17 cells in experimental autoimmune encephalomyelitis. *J Interferon Cytokine Res* (2011) **31**(7):575–87. doi:10.1089/jir.2010.0137
60. Yeh WI, McWilliams IL, Harrington LE. Autoreactive Tbet-positive CD4 T cells develop independent of classic Th1 cytokine signaling during experimental autoimmune encephalomyelitis. *J Immunol* (2011) **187**(10):4998–5006. doi:10.4049/jimmunol.1100031
61. Mangalam AK, Luo N, Luckey D, Papke L, Hubbard A, Wussow A, et al. Absence of IFN-gamma increases brain pathology in experimental autoimmune encephalomyelitis-susceptible DRB1*0301.DQ8 HLA transgenic mice through secretion of proinflammatory cytokine IL-17 and induction of pathogenic monocytes/microglia into the central nervous system. *J Immunol* (2014) **193**(10):4859–70. doi:10.4049/jimmunol.1302008
62. Yeh WI, McWilliams IL, Harrington LE. IFNgamma inhibits Th17 differentiation and function via Tbet-dependent and Tbet-independent mechanisms. *J Neuroimmunol* (2014) **267**(1–2):20–7. doi:10.1016/j.jneuroim.2013.12.001
63. Wildbaum G, Zohar Y, Karin N. Antigen-specific CD25- Foxp3- IFN-gamma(high) CD4+ T cells restrain the development of experimental allergic encephalomyelitis by suppressing Th17. *Am J Pathol* (2010) **176**(6):2764–75. doi:10.2353/ajpath.2010.090855
64. Ponomarev, Novikova M, Yassai M, Szczepanik M, Gorski J, Dittel BN. Gamma delta T cell regulation of IFN-gamma production by central nervous system-infiltrating encephalitogenic T cells: correlation with recovery from experimental autoimmune encephalomyelitis. *J Immunol* (2004) **173**(3):1587–95. doi:10.4049/jimmunol.173.3.1587
65. Wang Z, Hong J, Sun W, Xu G, Li N, Chen X, et al. Role of IFN-γ in induction of Foxp3 and conversion of CD4+CD25-T cells to CD4+ Tregs. *J Clin Invest* (2006) **116**:2434–41. doi:10.1172/JCI25826
66. Ortega SB, Kashi VP, Tyler AF, Cunnusamy K, Mendoza JP, Karandikar NJ. The disease-ameliorating function of autoregulatory CD8 T cells is mediated by targeting of encephalitogenic CD4 T cells in experimental autoimmune encephalomyelitis. *J Immunol* (2013) **191**(1):117–26. doi:10.4049/jimmunol.1300452
67. Chen ML, Yan BS, Kozoriz D, Weiner HL. Novel CD8+ Treg suppress EAE by TGF-beta- and IFN-gamma-dependent mechanisms. *Eur J Immunol* (2009) **39**(12):3423–35. doi:10.1002/eji.200939441
68. Tang X, Maricic I, Kumar V. Anti-TCR antibody treatment activates a novel population of nonintestinal CD8 alpha alpha+ TCR alpha beta+ regulatory T cells and prevents experimental autoimmune encephalomyelitis. *J Immunol* (2007) **178**(10):6043–50. doi:10.4049/jimmunol.178.10.6043
69. Tang X, Maricic I, Purohit N, Bakamjian B, Reed-Loisel LM, Beeston T, et al. Regulation of immunity by a novel population of Qa-1-restricted CD8alphaalpha+TCRalphabeta+ T cells. *J Immunol* (2006) **177**(11):7645–55. doi:10.4049/jimmunol.177.11.7645
70. Beeston T, Smith TR, Maricic I, Tang X, Kumar V. Involvement of IFN-gamma and perforin, but not Fas/FasL interactions in regulatory T cell-mediated suppression of experimental autoimmune encephalomyelitis. *J Neuroimmunol* (2010) **229**(1–2):91–7. doi:10.1016/j.jneuroim.2010.07.007
71. Bahri R, Bollinger A, Bollinger T, Orinska Z, Bulfone-Paus S. Ectonucleotidase CD38 demarcates regulatory, memory-like CD8+ T cells with IFN-gamma-mediated suppressor activities. *PLoS One* (2012) **7**(9):e45234. doi:10.1371/journal.pone.0045234
72. Tyler AF, Mendoza JP, Firan M, Karandikar NJ. CD8 T cells are required for glatiramer acetate therapy in autoimmune demyelinating disease. *PLoS One* (2013) **8**(6):e66772. doi:10.1371/journal.pone.0066772
73. Karandikar NJ, Crawford MP, Yan X, Ratts RB, Brenchley JM, Ambrozak DR, et al. Glatiramer acetate (Copaxone) therapy induces CD8(+) T cell responses in patients with multiple sclerosis. *J Clin Invest* (2002) **109**(5):641–9. doi:10.1172/JCI14380
74. Lee E, Chanamara S, Pleasure D, Soulika AM. IFN-gamma signaling in the central nervous system controls the course of experimental autoimmune encephalomyelitis independently of the localization and composition of inflammatory foci. *J Neuroinflammation* (2012) **9**:7. doi:10.1186/1742-2094-9-7
75. Willenborg DO, Staykova M, Fordham S, O'Brien N, Linares D. The contribution of nitric oxide and interferon gamma to the regulation of the neuro-inflammation in experimental autoimmune encephalomyelitis. *J Neuroimmunol* (2007) **191**(1–2):16–25. doi:10.1016/j.jneuroim.2007.09.007
76. Fenyk-Melody JE, Garrison AE, Brunnert SR, Weidner JR, Shen F, Shelton BA, et al. Experimental autoimmune encephalomyelitis is exacerbated in mice lacking the NOS2 gene. *J Immunol* (1998) **160**(6):2940–6.
77. Sahrbacher UC, Lechner F, Eugster HP, Frei K, Lassmann H, Fontana A. Mice with an inactivation of the inducible nitric oxide synthase gene are susceptible to experimental autoimmune encephalomyelitis. *Eur J Immunol* (1998) **28**(4):1332–8. doi:10.1002/(SICI)1521-4141(199804)28:04<1332::AID-IMMU1332>3.3.CO;2-7

78. Maghazachi AA. Role of natural killer cells in multiple sclerosis. *ISRN Immunol* (2012) **2012**:14. doi:10.5402/2012/795075
79. Segal BM. The role of natural killer cells in curbing neuroinflammation. *J Neuroimmunol* (2007) **191**(1–2):2–7. doi:10.1016/j.jneuroim.2007.09.006
80. Kaur G, Trowsdale J, Fugger L. Natural killer cells and their receptors in multiple sclerosis. *Brain* (2013) **136**(Pt 9):2657–76. doi:10.1093/brain/aws159
81. Takahashi K, Aranami T, Endoh M, Miyake S, Yamamura T. The regulatory role of natural killer cells in multiple sclerosis. *Brain* (2004) **127**(Pt 9):1917–27. doi:10.1093/brain/awh219
82. Zhang B, Yamamura T, Kondo T, Fujiwara M, Tabira T. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *J Exp Med* (1997) **186**(10):1677–87. doi:10.1084/jem.186.10.1677
83. Matsumoto Y, Kohyama K, Aikawa Y, Shin T, Kawazoe Y, Suzuki Y, et al. Role of natural killer cells and TCR gamma delta T cells in acute autoimmune encephalomyelitis. *Eur J Immunol* (1998) **28**(5):1681–8. doi:10.1002/(SICI)1521-4141(199805)28:05<1681::AID-IMMU1681>3.0.CO;2-T
84. Winkler-Pickett R, Young HA, Cherry JM, Diehl J, Wine J, Back T, et al. In vivo regulation of experimental autoimmune encephalomyelitis by NK cells: alteration of primary adaptive responses. *J Immunol* (2008) **180**(7):4495–506. doi:10.4049/jimmunol.180.7.4495
85. Morandi B, Bramanti P, Bonaccorsi I, Montalto E, Oliveri D, Pezzino G, et al. Role of natural killer cells in the pathogenesis and progression of multiple sclerosis. *Pharmacol Res* (2008) **57**(1):1–5. doi:10.1016/j.phrs.2007.11.003
86. Xu W, Fazekas G, Hara H, Tabira T. Mechanism of natural killer (NK) cell regulatory role in experimental autoimmune encephalomyelitis. *J Neuroimmunol* (2005) **163**(1–2):24–30. doi:10.1016/j.jneuroim.2005.02.011
87. Shi FD, Takeda K, Akira S, Sarvetnick N, Ljunggren HG. IL-18 directs autoreactive T cells and promotes autodestruction in the central nervous system via induction of IFN-gamma by NK cells. *J Immunol* (2000) **165**(6):3099–104. doi:10.4049/jimmunol.165.6.3099
88. Brady J, Hayakawa Y, Smyth MJ, Nutt SL. IL-21 induces the functional maturation of murine NK cells. *J Immunol* (2004) **172**(4):2048–58. doi:10.4049/jimmunol.172.4.2048
89. Strengell M, Matikainen S, Siren J, Lehtonen A, Foster D, Julkunen I, et al. IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells. *J Immunol* (2003) **170**(11):5464–9. doi:10.4049/jimmunol.170.11.5464
90. Vollmer TL, Liu R, Price M, Rhodes S, La Cava A, Shi FD. Differential effects of IL-21 during initiation and progression of autoimmunity against neuroantigen. *J Immunol* (2005) **174**(5):2696–701. doi:10.4049/jimmunol.174.5.2696
91. Xie ZX, Zhang HL, Wu XJ, Zhu J, Ma DH, Jin T. Role of the immunogenic and tolerogenic subsets of dendritic cells in multiple sclerosis. *Mediators Inflamm* (2015) **2015**:513295. doi:10.1155/2015/513295
92. Ganguly D, Haak S, Sisirak V, Reizis B. The role of dendritic cells in autoimmunity. *Nat Rev Immunol* (2013) **13**(8):566–77. doi:10.1038/nri3477
93. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* (2010) **28**:445–89. doi:10.1146/annurev-immunol-030409-101212
94. Barnes MJ, Powrie F. Hybrid Treg cells: steel frames and plastic exteriors. *Nat Immunol* (2009) **10**:563–4. doi:10.1038/ni0609-563
95. Kleinewietfeld M, Hafler DA. The plasticity of human Treg and Th17 cells and its role in autoimmunity. *Semin Immunol* (2013) **25**:305–12. doi:10.1016/j.smim.2013.10.009
96. Legroux L, Arbour N. Multiple sclerosis and T lymphocytes: an entangled story. *J Neuroimmune Pharmacol* (2015). doi:10.1007/s11481-015-9614-0
97. Kuchroo VK, Anderson AC, Waldner H, Munder M, Bettelli E, Nicholson LB. T cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire. *Annu Rev Immunol* (2002) **20**:101–23. doi:10.1146/annurev.immunol.20.081701.141316
98. Kurschus FC, Croxford AL, Heinen AP, Wortge S, Ielo D, Waisman A. Genetic proof for the transient nature of the Th17 phenotype. *Eur J Immunol* (2010) **40**(12):3336–46. doi:10.1002/eji.201040755
99. Hirota K, Duarte JH, Veldhoen M, Hornsby E, Li Y, Cua DJ, et al. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol* (2011) **12**(3):255–63. doi:10.1038/ni.1993
100. Paul S, Shilpi, Lal G. Role of gamma-delta (gammadelta) T cells in autoimmunity. *J Leukoc Biol* (2015) **97**(2):259–71. doi:10.1189/jlb.3RU0914-443R
101. Wohler JE, Smith SS, Zinn KR, Bullard DC, Barnum SR. Gammadelta T cells in EAE: early trafficking events and cytokine requirements. *Eur J Immunol* (2009) **39**(6):1516–26. doi:10.1002/eji.200839176
102. Rajan AJ, Klein JD, Brosnan CF. The effect of gammadelta T cell depletion on cytokine gene expression in experimental allergic encephalomyelitis. *J Immunol* (1998) **160**(12):5955–62.
103. Duhen T, Duhen R, Lanzavecchia A, Sallusto F, Campbell DJ. Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. *Blood* (2012) **119**(19):4430–40. doi:10.1182/blood-2011-11-392324
104. Daniel V, Sadeghi M, Wang H, Opelz G. CD4+CD25+Foxp3+IFN-gamma+ human induced T regulatory cells are induced by interferon-gamma and suppress alloresponses nonspecifically. *Hum Immunol* (2011) **72**(9):699–707. doi:10.1016/j.humimm.2011.05.020
105. Venigalla RK, Guttikonda PJ, Eckstein V, Ho AD, Sertel S, Lorenz HM, et al. Identification of a human Th1-like IFNgamma-secreting Treg subtype deriving from effector T cells. *J Autoimmun* (2012) **39**(4):377–87. doi:10.1016/j.jaut.2012.06.004
106. Koch Ma, Tucker-heard G, Perdue NR, Killebrew JR, Urdahl B, Campbell DJ. T-bet controls regulatory T cell homeostasis and function during type-1 inflammation. *Nat Immunol* (2009) **10**:595–602. doi:10.1038/ni.1731.T-bet
107. Koch Ma, Thomas KN, Perdue NR, Smigiel KS, Srivastava S, Campbell DJ. T-bet+ Treg cells undergo abortive Th1 cell differentiation due to impaired expression of IL-12 receptor β2. *Immunity* (2012) **37**:501–10. doi:10.1016/j.immuni.2012.05.031.T-bet
108. Zheng J, Liu Y, Qin G, Lam KT, Guan J, Xiang Z, et al. Generation of human Th1-like regulatory CD4+ T cells by an intrinsic IFN-gamma- and T-bet-dependent pathway. *Eur J Immunol* (2011) **41**(1):128–39. doi:10.1002/eji.201040724
109. Esposito M, Ruffini F, Bergami A, Garzetti L, Borsellino G, Battistini L, et al. IL-17- and IFN-γ-secreting Foxp3+ T cells infiltrate the target tissue in experimental autoimmunity. *J Immunol* (2010) **185**:7467–73. doi:10.4049/jimmunol.1001519
110. Dominguez-Villar M, Baecher-Allan CM, Hafler Da. Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease. *Nature medicine* (2011) **17**:673–5. doi:10.1038/nm.2389
111. Ford ML, Evavold BD. Specificity, magnitude, and kinetics of MOG-specific CD8+ T cell responses during experimental autoimmune encephalomyelitis. *Eur J Immunol* (2005) **35**(1):76–85. doi:10.1002/eji.200425660
112. Huseby ES, Liggitt D, Brabb T, Schnabel B, Ohlen C, Goverman J. A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. *J Exp Med* (2001) **194**(5):669–76. doi:10.1084/jem.194.5.669
113. Bettini M, Rosenthal K, Evavold BD. Pathogenic MOG-reactive CD8+ T cells require MOG-reactive CD4+ T cells for sustained CNS inflammation during chronic EAE. *J Neuroimmunol* (2009) **213**(1–2):60–8. doi:10.1016/j.jneuroim.2009.05.017
114. Sun D, Whitaker JN, Huang Z, Liu D, Coleclough C, Wekerle H, et al. Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. *J Immunol* (2001) **166**(12):7579–87. doi:10.4049/jimmunol.166.12.7579
115. York NR, Mendoza JP, Ortega SB, Benagh A, Tyler AF, Firan M, et al. Immune regulatory CNS-reactive CD8+T cells in experimental autoimmune encephalomyelitis. *J Autoimmun* (2010) **35**(1):33–44. doi:10.1016/j.jaut.2010.01.003
116. Smith TR, Kumar V. Revival of CD8+ Treg-mediated suppression. *Trends Immunol* (2008) **29**(7):337–42. doi:10.1016/j.it.2008.04.002
117. Bar-Or A, Fawaz L, Fan B, Darlington PJ, Rieger A, Ghorayeb C, et al. Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? *Ann Neurol* (2010) **67**(4):452–61. doi:10.1002/ana.21939

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Immune response modulation by vitamin D: role in systemic lupus erythematosus

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Vitamin D plays key roles as a natural immune modulator and has been implicated in the pathophysiology of autoimmune diseases, including systemic lupus erythematosus (SLE). This review presents a summary and analysis of the recent literature regarding immunoregulatory effects of vitamin D as well as its importance in SLE development, clinical severity, and possible effects of supplementation in disease treatment.

Keywords: vitamin D, systemic lupus erythematosus

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Introduction

1,25-Dihydroxyvitamin D is a steroid hormone, primarily known for its important role in calcium homeostasis (1). The description that several human tissues and cells express the vitamin D receptor (VDR), allows a growing interest in extra-skeletal functions of this vitamin (2). It is now clear that vitamin D plays an essential role in a variety of physiological conditions and that its deficiency is associated with chronic illnesses, including disorders of calcium metabolism, cancers, cardiovascular, and of our special interest, autoimmune diseases: applying to both development and severity of disease (3, 4). In this review, currently available data are summarized to give an overview of the role vitamin D plays on cells of the immune system and the regulation of inflammatory responses, with special emphasis on the role it has in the treatment of systemic lupus erythematosus (SLE).

Vitamin D comes from three potential sources: (i) it can be made in the skin from exposure to sunlight, (ii) nutritional sources, and (iii) supplements (5, 6). In humans, vitamin D is mainly synthesized in the skin after exposure to UVB whereas only a minor part (<10%) is derived from dietary sources (7). There are two major forms of vitamin D: ergocalciferol (vitamin D_2) that is obtained from UV irradiation and cholecalciferol (vitamin D_3) that is synthesized in the skin and is present in oil-rich fish (8). Both vitamin D_2 and vitamin D_3 are used for food fortification (such as dairy products) and in vitamin D supplements. Vitamin D levels depend on season, reaching their lowest levels after winter and their maximum at the end of summer.

Vitamin D (D represents D_2, or D_3, or both) after it is ingested is incorporated into chylomicrons, which are absorbed into the lymphatic system and enter the venous blood. In the skin, cholecalciferol is synthesized from 7-dehydrocholesterol when exposed to UVB. Vitamin D that comes from the skin or diet is biologically inert and requires its first hydroxylation in the liver by the vitamin D-25-hydroxylase (25-OHase) to 25(OH)D, which represents the main circulating vitamin D metabolite and is the most reliable parameter to define human vitamin D status (9). However, 25(OH)D requires a further hydroxylation in the kidneys by the 25(OH)D-1-OHase (CYP27B1) to form the biologically active form of vitamin D 1,25(OH)2D (9). This process is under strict control of parathyroid hormone and the phosphaturic hormone fibroblast growth factor 23 (FGF-23). High levels of vitamin D inhibit CYP27B1 and stimulate CYP24A1, an enzyme that metabolizes vitamin D into the

inactive, water-soluble form, calcitroic acid, which is then excreted into the bile. Circulating levels of 1,25(OH)2D are determined by renal CYP27B1 activity. Interestingly, other cell types, including immune cells, also express CYP27B1, and these cells are able to convert the inactive hormone into the active form, in an autocrine or paracrine manner. This process lacks feedback mechanisms (as the ones described for kidney cells), and allows the production of high local concentrations of vitamin D.

1,25(OH)2D interacts with VDR, which is present in several human tissues and cells (1, 9). VDR is a member of transcription factor family, characterized by a highly conserved DNA-binding domain and a structurally conserved ligand-binding domain, and it acts as a modulator of gene transcription (1). 1,25(OH)2D may be responsible for regulating up to 200 genes that may facilitate many of the pleiotropic health benefits that have been reported for vitamin D (1, 5, 6). Ligand binding initiates a conformational change that increases the receptor's affinity to the retinoid X receptor (RXR), then VDR-vitamin D complex forms heterodimers with RXR and the complex binds to vitamin D response elements on DNA and recruits a number of nuclear co-activator and co-repressor proteins (10). The gene encoding VDR is located on chromosome 12q13.11, contains 9 exons and 8 introns and several single nucleotide polymorphisms (SNPs) have been described. Mainly four, including *Bsm*I and ApaI (both in intron 8), Fok1 and *Taq*I (located in the start codon), have been intensively studied (11).

Serum 25(OH)D is considered as the most accurate marker for vitamin D. Vitamin D deficiency has been recently recommended by the Institute of Medicine (IOM) as a vitamin D of $<$20 ng/mL, whereas vitamin D insufficiency has been defined as levels between 21 and 29 ng/mL (12, 13). This classification is based on vitamin D effects on bone and mineral homeostasis. The serum concentration of the active 1,25(OH)2D is approximately 1000-fold lower and far below the effective concentration described in *in vitro* studies. Most *in vitro* studies use more that 100-fold higher concentrations of 1,25(OH)2D than found in serum, to obtain an effect. It has been suggested that the level of circulating 1,25(OH)2D is too low to affect immune responses *in vivo*, and that sufficient levels are obtained by local conversion of 25(OH)D3 to 1,25(OH)2D. Other important players influencing the bioavailable levels of vitamin D are the vitamin D-binding protein (DBP) and albumin. 25(OH)D3 and 1,25(OH)2D circulate bound to DBP (85–90%) and albumin (10–15%) with $<$1% in their free form (14). Studies in mice lacking DBP have shown that DBP acts as a vitamin D reservoir by protecting 25(OH)D3 and 1,25(OH)2D from degradation and renal secretion (15).

The major cause of vitamin D deficiency is inadequate exposure to sunlight (6, 16). There is an inverse association of serum vitamin D and body mass index (BMI), and thus, obesity is also associated with vitamin D deficiency (17). Patients with fat malabsorption syndromes and bariatric patients are often unable to absorb the fat-soluble vitamin D, and patients with nephritic syndrome lose 25(OH)D bound to vitamin DBP in the urine (18). Patients on a wide variety of medications, chronic granuloma-forming disorders, some lymphomas, and primary hyperparathyroidism have a high risk for vitamin D deficiency (19).

Supplementation with vitamin D in the general population has shown fracture prevention, suggested benefit in cardiovascular health, colorectal cancer prevention and reduction of proteinuria in patients with chronic kidney disease (20, 21). However, a possible harm of vitamin D supplementation has been documented in some studies: a meta-analysis showed that supplementation with calcium and vitamin D could be associated with the modest increase in the risk of cardiovascular events, especially myocardial infarction (22). Currently, no international consensus is available on the optimal vitamin D supplementation level; recommendations differ in many countries and medical societies. The Endocrine Society considers a supplementation of 10,000 IU daily to be safe, the IOM considers 4000 IU/day and the European Food and Safety Authority recommends staying below 4000 IU/day. The most common forms of vitamin D for supplementation are cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2), although administration of calcitriol is limited because of potential side effects. A recent work by Souberbielle et al. (23) has shown that a target level of at least 30–40 ng/mL of vitamin D serum level was recommended in adult patients with risk of fractures, falls, cancers, and autoimmune and cardiovascular disease. Serum levels higher than 150 ng/mL may cause acute vitamin D intoxication with hypercalcemia, hypercalciuria, and calcifications in different organs.

Vitamin D and the Immune Response

Several studies suggest that calcitriol can enhance the innate immune response, whereas it can inhibit the adaptive immune response (24). Early evidence suggesting that vitamin D could act as stimulant for innate immunity comes from reports about tuberculosis treatment with cod liver oil (25). It enhances chemotaxis and phagocytic capabilities of innate immune cells and activates the transcription of antimicrobial peptides, such as defensin B and cathelicidin (26). Low 25(OH)D concentrations have been linked to increased mortality caused by severe infections in end-stage renal disease patients, and have been associated with upper respiratory tract infections and allergic asthma.

In particular, dendritic cells (DCs) are important targets for the immunomodulatory effects of vitamin D. DCs are professional antigen presenting cells (APCs) that play an important role in maintaining peripheral tolerance by preventing self-reactive T cells from causing autoimmune damage. Through their unique ability to efficiently capture antigens and trigger the adaptive immune response, DCs are critical for the defense against infectious agents and tumors (27). In addition to activating immune responses, DCs also play a central role in peripheral T cell tolerance, by inducing T cell anergy or unresponsiveness to self-antigens (28). Calcitriol and its analogs are able to suppress DC differentiation (29) and maturation *in vitro*. Likewise, vitamin D, by inhibiting the maturation of DCs, can make them tolerogenic (30–32). It has been shown that DC can produce 1,25(OH)2D from 25(OH)D *in vitro*, and respond to this through the VDR in an autocrine fashion. Since DCs are central to the maintenance of self-tolerance, it is possible that a deficiency in vitamin D could have consequences on their maturation and function and consequently on the risk of developing autoimmune diseases as

well as disease severity. In addition, vitamin D exerts effects that oppose the effect of IL-4 on MHC class II antigen expression in human monocytes and specifically modulates human monocyte phenotype and function by altering HLA-DR expression and antigen presentation, leaving lytic function intact (33).

T and B cells express VDR and are important target cells of calcitriol immune regulation. Vitamin D can suppress cellular and humoral immunity in several animal models as it plays an important role in regulating proliferation, differentiation of activated B cell, and immunoglobulin production (34, 35). *In vitro* studies have shown that vitamin D inhibits IL-17 synthesis, inhibiting Th17 differentiation and increases the quantity of $CD4^+$ $CD25^+$ T regulatory cells, which produce IL-10 and amplifies a Th1–Tr1 switch (36) (**Figure 1**).

Vitamin D and Autoimmune Diseases

Several studies have now reported vitamin D insufficiencies in various autoimmune disorders. In addition to observational studies, numerous randomized trials have addressed the question whether vitamin D levels are associated with the risk of developing autoimmunity and whether development and disease progression can be influenced by vitamin D supplementation. In the following section, we have summarized the latest results associating vitamin D insufficiency and vitamin D supplementation in SLE. If causal, these associations might be of great importance for public health.

Vitamin D and Systemic Lupus Erythematosus

Systemic lupus erythematosus is a chronic multisystem autoimmune disease that can manifest with a diverse array of clinical symptoms and which is characterized by the production of autoantibodies directed against nuclear antigens (37). Systemic injury may arise as a consequence of inflammation caused by direct autoantibody-mediated tissue injury and the deposition of complement-fixing immune complexes (ICs) (38). IC-mediated inflammation has been shown to damage multiple organs, such as skin, joints, kidneys, brain, and blood vessels. Cellular and molecular mechanisms underlying this autoimmune disease are not completely understood. Currently, there is no cure for SLE, and treatments, such as long-term corticosteroids, may contribute to further health risks.

In SLE animal models, it has been shown a relation between vitamin D and disease manifestations. Lemire et al. (39) showed that in the MRL/1 SLE mouse model, supplementation with 25(OH)D for 18 weeks reduced dermatologic lesions, proteinuria, and anti-DNA antibodies. Instead, Vaisberg et al. (40) described opposing results in the NZB/W mice that were injected with different concentrations of vitamin D. Treatment with cholecalciferol led to a worsening of the histopathological findings in the kidneys of female F1 NZB/W mice. *In vitro* studies of SLE-derived PBMCs have shown that when these cells are incubated with calcitriol, reduced cellular proliferation and anti-DNA antibodies are observed (41). Also, *in vitro* vitamin D reduced the expression of CD40, MHC class II, CD86, and inhibited the activation of APCs

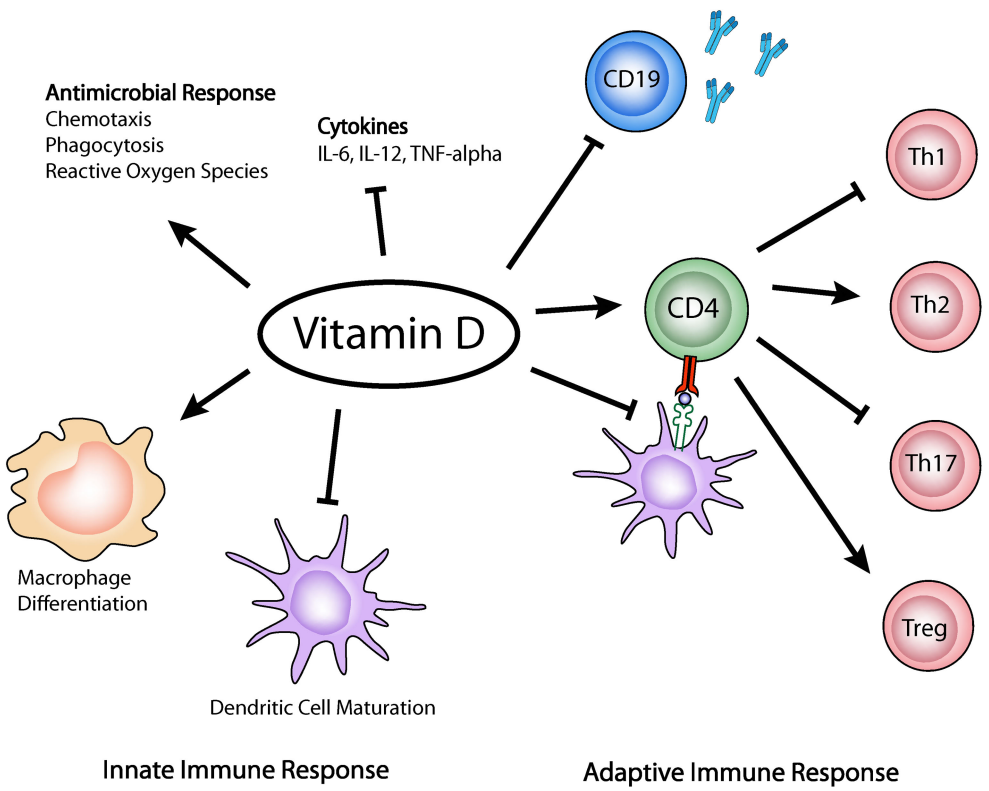

FIGURE 1 | Vitamin D effects on the innate and adaptive immune response. Vitamin D has been shown to enhance chemotaxis, antimicrobial peptides, and macrophage differentiation. It can also inhibit DCs maturation, Th1 and Th17 differentiation, and promotes immunoregulatory functions of Treg cells.

TABLE 1 | Clinical studies of the effects of vitamin D supplementation in patients with systemic lupus erythematosus.

Author, year, country	Sample size	Subjects	Intervention (type, dose, duration)	Study results
Petri, 2013, USA (64)	1006	SLE	Oral cholecalciferol 50,000 IU weekly + 200 U calcium/vitamin D twice daily	A 20-U increase in the 25(OH)D level was associated with decrease of 0.22 of SELENA-SLEDAI and 2% decrease in urine protein-to-creatinine ratio
Abou-Raya, 2013, Egypt (71)	267	SLE	Oral cholecalciferol 2000 IU/day or placebo for 12 months	69% suboptimal VitD 39% deficient VitD Lower VitD correlates with higher disease activity Change in 25(OH)D after 12 months is associated with improvement in inflammatory-hemostatic markers
Ruiz-Irastorza, 2010, Spain (70)	80	SLE	Oral cholecalciferol 600–800 IU day for 24 months	Beneficial effect on fatigue, no significant correlations were seen in SLEDAI or SDI values
Lima, 2015, Brazil	60	juvSLE	Oral cholecalciferol 50,000 IU/week or placebo for 24 weeks	Beneficial effect on fatigue and decrease disease activity
Aranow, 2015, USA (73)	57	SLE	Oral cholecalciferol 2000 or 4000 IU for 12 weeks	No changes in IFN signature in vitamin D deficient SLE patients
Andreoli, 2015, Italy (72)	34	SLE	Oral cholecalciferol Intensive R: 300,000 IU initial bolus followed by 50,000 IU monthly (850,000 annually) Standard R: 25,000 IU monthly (300,000 annually) for 12 months and then switched in the second year	Intensive regimen significantly raise vitamin D serum levels No significant differences in disease activity, or SLE serology were found
Piantoni, 2015, Italy (74)	34	SLE	Oral cholecalciferol Intensive R: 300,000 IU initial bolus followed by 50,000 IU monthly (850,000 annually) Standard R: 25,000 IU monthly (300,000 annually) for 12 months and then switched in the second year	Vitamin D treatment promotes regulatory T cells proliferation and production of Th2 cytokines
Terrier, 2012, France (75)	20	SLE	Oral cholecalciferol 100,000 IU/week during 4 weeks 100,000 IU/month for 6 months	Increase of naive CD4 T cells Increase in regulatory T cells Decrease Th1 and Th17 cells Decrease memory B cells Decrease anti-DNA antibodies

derived from SLE patients (42). Ben-Zvi et al. (43) showed that vitamin D treatment reduced the expression of IFNα-regulated genes in healthy and SLE patients-derived DCs in response to factors in activating SLE plasma.

Levels of micro RNA (miRNA)-146a in PBMCs correlate significantly with disease activity in SLE patients and urinary expression correlates with estimated glomerular filtration rate (44). Recent studies have shown that after treatment with vitamin D, miRNA-146a levels of SLE patients tend to decrease (44).

Association studies with VDR polymorphism and SLE susceptibility have been performed in different populations with controversial results (45–47). A meta-analysis study, including a total of 11 case-control studies (8 from Asian, 2 European, 1 Latino population), of 1683 patients and 1883 healthy individuals revealed associations between the VDR polymorphisms and SLE (48). These findings show that the *Bsm*I and *Fok*I polymorphisms are associated with increased risk of SLE, especially in the Asian population. Limitations to the study are the low representation of European and Latino populations.

Hypovitaminosis D is highly prevalent in SLE as a result of avoidance of sunshine, renal insufficiency, and the use of medications, such as glucocorticoids, anticonvulsants, and antimalarials, which alter the metabolism of vitamin D, vitamin D-binding protein levels or downregulate the functions of the VDR (49, 50). Several studies have reported suboptimal vitamin D levels in patients with SLE, whose prevalence described varies between 36.8% and 75% in the different populations studied (49, 51–54). Low levels of vitamin D have been correlated with disease activity, and are associated with osteoporosis, fatigue, and certain cardiovascular risk factors in SLE patients (49, 55). The wide variation reported can be related to age, ethnicity, geographic location, and season at the time of the study.

Vitamin D Deficiency and Disease Activity

There are several cross-sectional studies examining the relationship between low vitamin D levels and SLE activity. It has been shown that vitamin D deficiency has an association with disease activity and some clinical manifestations, but there are discrepancies between the different populations studied. No associations between adolescent dietary vitamin D intake and adult SLE risk were observed in a prospective cohort of women (56, 57). Mok et al. reported in a cross-sectional study with 290 Chinese patients with SLE, that vitamin D deficiency was inversely correlated with disease activity, measured by SLEDAI scores (58), and in another study, they reported associations with anti-dsDNA levels (59). In addition, Lertratanakul et al. showed that lower baseline vitamin D levels are associated with higher cardiovascular risk factors and more active SLE (60). Besides, SLE patients with higher vitamin D levels were less likely to have hypertension and hyperlipidemia. A recent study that evaluated 129 Indian patients

with SLE found a negative correlation of vitamin D levels with disease activity, anti-dsDNA, plasma IFN-α, and IFN-α gene expression (61). Baseline vitamin D levels were not associated with relapse-free survival rate (62). In Australian patients, Yap et al. showed that low vitamin D is associated with higher disease activity, and an increase in vitamin D was associated with reduced disease activity over time (50). Other studies have shown that vitamin D deficiency is associated with a higher B cell activation, more frequent leukopenia or renal involvement with proteinuria, and higher titers of anti-DNA (51, 63–66). By contrast, others studies have reported no association between vitamin D and fatigue, SLEDAI score or cytokine profile (67–69).

Overall, both *in vitro* and *in vivo* studies of vitamin D effects in SLE provide immunological basis for potential beneficial effects of vitamin D in this disease. Despite all this overwhelming evidence favoring the use of vitamin D in SLE, to date, vitamin D supplementation is not the standard of care for patients with SLE. Interventional studies have been reported (see **Table 1**), with the purpose of changing clinical outcomes; however, results are still not conclusive.

Ruiz-Irastorza et al. showed no significant correlations with SLE clinical activity, evaluating SLEDAI or SDI values, and suggested that increasing vitamin D levels may have a beneficial effect on fatigue (70). Petri et al. studied a prospective cohort of 1006 patients receiving supplementation with 50,000 IU weekly for 128 weeks. Results showed that vitamin D increases were associated with a decrease in disease activity and proteinuria (64). Also, Abou-Raya et al. reported that lower vitamin D levels correlated with disease activity and improvement in inflammatory and hemostatic parameters was observed after 12 months treatment (71). Latest studies have found no association between supplementation and disease activity and no changes in IFN signature in vitamin D deficient SLE patients (72, 73). Other studies have shown effects in cytokine profiles and T cell differentiation (74, 75).

A recent systematic review and meta-analyses of observational and randomized trials (76) found no convincing evidence of a clear role of vitamin D with highly significant results in both randomized and observational studies. The number of randomized, controlled trials with vitamin D is scarce, so more data are needed to reach a conclusion. Also, the effects of multiple compounds when administered simultaneously, and the follow-up time, may be inadequate to allow differences in disease occurrence. More efforts are required in order to clarify the role that vitamin D has in this disease, to regulate the type of supplementation required, and to determine the minimal beneficial levels. Specific attention to maintaining optimal vitamin D levels may be beneficial in the management of SLE.

Conclusion

Vitamin D exerts important regulatory functions on cells from the innate as well as from the adaptive immune response. Indeed, accumulating evidence has shown that insufficient vitamin D levels may lead to dysregulation of immune responses, and thus contribute to autoimmune diseases. There is no consensus about recommended targeted serum levels and the optimal mode and dose of vitamin D supplementation. It seems that higher doses for supplementation could have better outcomes in disease activity, but still there is great variability between studies and no conclusions can be obtained. More and larger studies are needed to determine how vitamin D supplementation affects the pathophysiology of SLE and how it may contribute to better efficacy of actual therapies.

Author Contributions

PB, RN, DH, and MI contributed to the design of this work, drafting, and revising it critically. All authors gave their final approval to the manuscript.

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References

1. Adams JS, Hewison M. Update in vitamin D. *J Clin Endocrinol Metab* (2010) **95**(2):471–8. doi:10.1210/jc.2009-1773
2. Gröber U, Spitz J, Reichrath J, Kisters K, Holick MF. Vitamin D: update 2013: from rickets prophylaxis to general preventive healthcare. *Dermatoendocrinol* (2013) **5**(3):331–47. doi:10.4161/derm.26738
3. Holick MF. Vitamin D deficiency. *N Engl J Med* (2007) **357**(3):266–81. doi:10.1056/NEJMra070553
4. Holick MF. Vitamin D: important for prevention of osteoporosis, cardiovascular heart disease, type 1 diabetes, autoimmune diseases, and some cancers. *South Med J* (2005) **98**(10):1024–7. doi:10.1097/01.SMJ.0000140865.32054.DB
5. Holick MF, Chen TC, Lu Z, Sauter E. Vitamin D and skin physiology: a D-lightful story. *J Bone Miner Res* (2007) **22**(Suppl 2):V28–33. doi:10.1359/jbmr.07s211
6. Moan J, Porojnicu AC, Dahlback A, Setlow RB. Addressing the health benefits and risks, involving vitamin D or skin cancer, of increased sun exposure. *Proc Natl Acad Sci U S A* (2008) **105**(2):668–73. doi:10.1073/pnas.0710615105
7. Holick MF. Vitamin D: a millenium perspective. *J Cell Biochem* (2003) **88**(2):296–307. doi:10.1002/jcb.10338
8. Prietl B, Treiber G, Pieber TR, Amrein K. Vitamin D and immune function. *Nutrients* (2013) **5**(7):2502–21. doi:10.3390/nu5072502
9. DeLuca HF. Overview of general physiologic features and functions of vitamin D. *Am J Clin Nutr* (2004) **80**(6 Suppl):1689S–96S.
10. Carlberg C, Campbell MJ. Vitamin D receptor signaling mechanisms: integrated actions of a well-defined transcription factor. *Steroids* (2013) **78**(2):127–36. doi:10.1016/j.steroids.2012.10.019
11. Yang CY, Leung PS, Adamopoulos IE, Gershwin ME. The implication of vitamin D and autoimmunity: a comprehensive review. *Clin Rev Allergy Immunol* (2013) **45**(2):217–26. doi:10.1007/s12016-013-8361-3
12. Rosen CJ, Gallagher JC. The 2011 IOM report on vitamin D and calcium requirements for North America: clinical implications for providers treating patients with low bone mineral density. *J Clin Densitom* (2011) **14**(2):79–84. doi:10.1016/j.jocd.2011.03.004
13. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab* (2011) **96**(1):53–8. doi:10.1210/jc.2010-2704
14. Kongsbak M, von Essen MR, Levring TB, Schjerling P, Woetmann A, Ødum N, et al. Vitamin D-binding protein controls T cell responses to vitamin D. *BMC Immunol* (2014) **15**:35. doi:10.1186/s12865-014-0035-2

15. Safadi FF, Hermey DC, Popoff SN, Seifert MF. Skeletal resistance to 1,25-dihydroxyvitamin D3 in osteopetrotic rats. *Endocrine* (1999) **11**(3):309–19. doi:10.1385/ENDO:11:3:309
16. Holick MF, Siris ES, Binkley N, Beard MK, Khan A, Katzer JT, et al. Prevalence of vitamin D inadequacy among postmenopausal North American women receiving osteoporosis therapy. *J Clin Endocrinol Metab* (2005) **90**(6):3215–24. doi:10.1210/jc.2004-2364
17. Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. *Am J Clin Nutr* (2000) **72**(3):690–3.
18. Dusso AS, Brown AJ, Slatopolsky E. Vitamin D. *Am J Physiol Renal Physiol* (2005) **289**(1):F8–28. doi:10.1152/ajprenal.00336.2004
19. Grey A, Lucas J, Horne A, Gamble G, Davidson JS, Reid IR. Vitamin D repletion in patients with primary hyperparathyroidism and coexistent vitamin D insufficiency. *J Clin Endocrinol Metab* (2005) **90**(4):2122–6. doi:10.1210/jc.2004-1772
20. Bischoff-Ferrari HA, Shao A, Dawson-Hughes B, Hathcock J, Giovannucci E, Willett WC. Benefit-risk assessment of vitamin D supplementation. *Osteoporos Int* (2010) **21**(7):1121–32. doi:10.1007/s00198-009-1119-3
21. Bolland MJ, Bacon CJ, Horne AM, Mason BH, Ames RW, Wang TK, et al. Vitamin D insufficiency and health outcomes over 5 y in older women. *Am J Clin Nutr* (2010) **91**(1):82–9. doi:10.3945/ajcn.2009.28424
22. Bolland MJ, Grey A, Avenell A, Gamble GD, Reid IR. Calcium supplements with or without vitamin D and risk of cardiovascular events: reanalysis of the women's health initiative limited access dataset and meta-analysis. *BMJ* (2011) **342**:d2040. doi:10.1136/bmj.d2040
23. Souberbielle JC, Body JJ, Lappe JM, Plebani M, Shoenfeld Y, Wang TJ, et al. Vitamin D and musculoskeletal health, cardiovascular disease, autoimmunity and cancer: recommendations for clinical practice. *Autoimmun Rev* (2010) **9**(11):709–15. doi:10.1016/j.autrev.2010.06.009
24. Lagishetty V, Misharin AV, Liu NQ, Lisse TS, Chun RF, Ouyang Y, et al. Vitamin D deficiency in mice impairs colonic antibacterial activity and predisposes to colitis. *Endocrinology* (2010) **151**(6):2423–32. doi:10.1210/en.2010-0089
25. Grad R. Cod and the consumptive: a brief history of cod-liver oil in the treatment of pulmonary tuberculosis. *Pharm Hist* (2004) **46**(3):106–20.
26. Scherberich JE, Kellermeyer M, Ried C, Hartinger A. 1-Alpha-calcidol modulates major human monocyte antigens and toll-like receptors TLR 2 and TLR4 in vitro. *Eur J Med Res* (2005) **10**(4):179–82.
27. Steinman RM. Decisions about dendritic cells: past, present, and future. *Annu Rev Immunol* (2012) **30**:1–22. doi:10.1146/annurev-immunol-100311-102839
28. Iruretagoyena MI, Wiesendanger M, Kalergis AM. The dendritic cell-T cell synapse as a determinant of autoimmune pathogenesis. *Curr Pharm Des* (2006) **12**(2):131–47. doi:10.2174/138161206775193145
29. Gordon JR, Ma Y, Churchman L, Gordon SA, Dawicki W. Regulatory dendritic cells for immunotherapy in immunologic diseases. *Front Immunol* (2014) **5**:7. doi:10.3389/fimmu.2014.00007
30. Adorini L, Amuchastegui S, Corsiero E, Laverny G, Le Meur T, Penna G. Vitamin D receptor agonists as anti-inflammatory agents. *Expert Rev Clin Immunol* (2007) **3**(4):477–89. doi:10.1586/1744666X.3.4.477
31. Griffin MD, Lutz W, Phan VA, Bachman LA, McKean DJ, Kumar R. Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo. *Proc Natl Acad Sci U S A* (2001) **98**(12):6800–5. doi:10.1073/pnas.121172198
32. Penna G, Amuchastegui S, Laverny G, Adorini L. Vitamin D receptor agonists in the treatment of autoimmune diseases: selective targeting of myeloid but not plasmacytoid dendritic cells. *J Bone Miner Res* (2007) **22**(Suppl 2):V69–73. doi:10.1359/jbmr.07s217
33. Rigby WF, Waugh M, Graziano RF. Regulation of human monocyte HLA-DR and CD4 antigen expression, and antigen presentation by 1,25-dihydroxyvitamin D3. *Blood* (1990) **76**(1):189–97.
34. Rolf L, Muris AH, Hupperts R, Damoiseaux J. Vitamin D effects on B cell function in autoimmunity. *Ann N Y Acad Sci* (2014) **1317**:84–91. doi:10.1111/nyas.12440
35. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. *J Immunol* (2007) **179**(3):1634–47. doi:10.4049/jimmunol.179.3.1634
36. Hayes CE, Hubler SL, Moore JR, Barta LE, Praska CE, Nashold FE. Vitamin D actions on CD4(+) T cells in autoimmune disease. *Front Immunol* (2015) **6**:100. doi:10.3389/fimmu.2015.00100
37. Croker JA, Kimberly RP. SLE: challenges and candidates in human disease. *Trends Immunol* (2005) **26**(11):580–6. doi:10.1016/j.it.2005.09.001
38. Hahn BH. Antibodies to DNA. *N Engl J Med* (1998) **338**(19):1359–68. doi:10.1056/NEJM199805073381906
39. Lemire JM, Ince A, Takashima M. 1,25-Dihydroxyvitamin D3 attenuates the expression of experimental murine lupus of MRL/l mice. *Autoimmunity* (1992) **12**(2):143–8. doi:10.3109/08916939209150321
40. Vaisberg MW, Kaneno R, Franco MF, Mendes NF. Influence of cholecalciferol (vitamin D3) on the course of experimental systemic lupus erythematosus in F1 (NZBxW) mice. *J Clin Lab Anal* (2000) **14**(3):91–6. doi:10.1002/(SICI)1098-2825(2000)14:3<91::AID-JCLA2>3.0.CO;2-O
41. Linker-Israeli M, Elstner E, Klinenberg JR, Wallace DJ, Koeffler HP. Vitamin D(3) and its synthetic analogs inhibit the spontaneous in vitro immunoglobulin production by SLE-derived PBMC. *Clin Immunol* (2001) **99**(1):82–93. doi:10.1006/clim.2000.4998
42. Lerman M, Burnham J, Behrens E. 1,25 Dihydroxyvitamin D3 limits monocyte maturation in lupus sera. *Lupus* (2011) **20**(7):749–53. doi:10.1177/0961203310394542
43. Ben-Zvi I, Aranow C, Mackay M, Stanevsky A, Kamen DL, Marinescu LM, et al. The impact of vitamin D on dendritic cell function in patients with systemic lupus erythematosus. *PLoS One* (2010) **5**(2):e9193. doi:10.1371/journal.pone.0009193
44. Wang G, Tam LS, Kwan BC, Li EK, Chow KM, Luk CC, et al. Expression of miR-146a and miR-155 in the urinary sediment of systemic lupus erythematosus. *Clin Rheumatol* (2012) **31**(3):435–40. doi:10.1007/s10067-011-1857-4
45. de Azevêdo Silva J, Monteiro Fernandes K, Trés Pancotto JA, Sotero Fragoso T, Donadi EA, Crovella S, et al. Vitamin D receptor (VDR) gene polymorphisms and susceptibility to systemic lupus erythematosus clinical manifestations. *Lupus* (2013) **22**(11):1110–7. doi:10.1177/0961203313500549
46. Carvalho C, Marinho A, Leal B, Bettencourt A, Boleixa D, Almeida I, et al. Association between vitamin D receptor (VDR) gene polymorphisms and systemic lupus erythematosus in Portuguese patients. *Lupus* (2015) **24**(8):846–53. doi:10.1177/0961203314566636
47. Mao S, Huang S. Association between vitamin D receptor gene BsmI, FokI, ApaI and TaqI polymorphisms and the risk of systemic lupus erythematosus: a meta-analysis. *Rheumatol Int* (2014) **34**(3):381–8. doi:10.1007/s00296-013-2898-6
48. Xiong J, He Z, Zeng X, Zhang Y, Hu Z. Association of vitamin D receptor gene polymorphisms with systemic lupus erythematosus: a meta-analysis. *Clin Exp Rheumatol* (2014) **32**(2):174–81.
49. Ruiz-Irastorza G, Egurbide MV, Olivares N, Martinez-Berriotxoa A, Aguirre C. Vitamin D deficiency in systemic lupus erythematosus: prevalence, predictors and clinical consequences. *Rheumatology (Oxford)* (2008) **47**(6):920–3. doi:10.1093/rheumatology/ken121
50. Yap KS, Morand EF. Vitamin D and systemic lupus erythematosus: continued evolution. *Int J Rheum Dis* (2015) **18**(2):242–9. doi:10.1111/1756-185X.12489
51. Ritterhouse LL, Crowe SR, Niewold TB, Kamen DL, Macwana SR, Roberts VC, et al. Vitamin D deficiency is associated with an increased autoimmune response in healthy individuals and in patients with systemic lupus erythematosus. *Ann Rheum Dis* (2011) **70**(9):1569–74. doi:10.1136/ard.2010.148494
52. Wright TB, Shults J, Leonard MB, Zemel BS, Burnham JM. Hypovitaminosis D is associated with greater body mass index and disease activity in pediatric systemic lupus erythematosus. *J Pediatr* (2009) **155**(2):260–5. doi:10.1016/j.jpeds.2009.02.033
53. Kamen DL, Cooper GS, Bouali H, Shaftman SR, Hollis BW, Gilkeson GS. Vitamin D deficiency in systemic lupus erythematosus. *Autoimmun Rev* (2006) **5**(2):114–7. doi:10.1016/j.autrev.2005.05.009
54. Huisman AM, White KP, Algra A, Harth M, Vieth R, Jacobs JW, et al. Vitamin D levels in women with systemic lupus erythematosus and fibromyalgia. *J Rheumatol* (2001) **28**(11):2535–9.
55. Mok CC. Vitamin D and systemic lupus erythematosus: an update. *Expert Rev Clin Immunol* (2013) **9**(5):453–63. doi:10.1586/eci.13.19
56. Costenbader KH, Feskanich D, Holmes M, Karlson EW, Benito-Garcia E. Vitamin D intake and risks of systemic lupus erythematosus and rheumatoid arthritis in women. *Ann Rheum Dis* (2008) **67**(4):530–5. doi:10.1136/ard.2007.072736
57. Hiraki LT, Munger KL, Costenbader KH, Karlson EW. Dietary intake of vitamin D during adolescence and risk of adult-onset systemic lupus erythematosus and rheumatoid arthritis. *Arthritis Care Res (Hoboken)* (2012) **64**(12):1829–36. doi:10.1002/acr.21776

58. Mok CC, Birmingham DJ, Ho LY, Hebert LA, Song H, Rovin BH. Vitamin D deficiency as marker for disease activity and damage in systemic lupus erythematosus: a comparison with anti-dsDNA and anti-C1q. *Lupus* (2012) **21**(1):36–42. doi:10.1177/0961203311422094
59. Mok CC, Birmingham DJ, Leung HW, Hebert LA, Song H, Rovin BH. Vitamin D levels in Chinese patients with systemic lupus erythematosus: relationship with disease activity, vascular risk factors and atherosclerosis. *Rheumatology (Oxford)* (2012) **51**(4):644–52. doi:10.1093/rheumatology/ker212
60. Lertratanakul A, Wu P, Dyer A, Urowitz M, Gladman D, Fortin P, et al. 25-Hydroxyvitamin D and cardiovascular disease in patients with systemic lupus erythematosus: data from a large international inception cohort. *Arthritis Care Res (Hoboken)* (2014) **66**(8):1167–76. doi:10.1002/acr.22291
61. Mandal M, Tripathy R, Panda AK, Pattanaik SS, Dakua S, Pradhan AK, et al. Vitamin D levels in Indian systemic lupus erythematosus patients: association with disease activity index and interferon alpha. *Arthritis Res Ther* (2014) **16**(1):R49. doi:10.1186/ar4479
62. Schoindre Y, Jallouli M, Tanguy ML, Ghillani P, Galicier L, Aumaître O, et al. Lower vitamin D levels are associated with higher systemic lupus erythematosus activity, but not predictive of disease flare-up. *Lupus Sci Med* (2014) **1**(1):e000027. doi:10.1136/lupus-2014-000027
63. Bogaczewicz J, Sysa-Jedrzejowska A, Arkuszewska C, Zabek J, Kontny E, McCauliffe D, et al. Vitamin D status in systemic lupus erythematosus patients and its association with selected clinical and laboratory parameters. *Lupus* (2012) **21**(5):477–84. doi:10.1177/0961203311427549
64. Petri M, Bello KJ, Fang H, Magder LS. Vitamin D in systemic lupus erythematosus: modest association with disease activity and urine protein/creatinine ratio. *Arthritis Rheum* (2013) **65**(7):1865–71. doi:10.1002/art.37953
65. Reynolds JA, Haque S, Berry JL, Pemberton P, Teh LS, Ho P, et al. 25-Hydroxyvitamin D deficiency is associated with increased aortic stiffness in patients with systemic lupus erythematosus. *Rheumatology (Oxford)* (2012) **51**(3):544–51. doi:10.1093/rheumatology/ker352
66. Toloza SM, Cole DE, Gladman DD, Ibañez D, Urowitz MB. Vitamin D insufficiency in a large female SLE cohort. *Lupus* (2010) **19**(1):13–9. doi:10.1177/0961203309345775
67. Souto M, Coelho A, Guo C, Mendonça L, Argolo S, Papi J, et al. Vitamin D insufficiency in Brazilian patients with SLE: prevalence, associated factors, and relationship with activity. *Lupus* (2011) **20**(10):1019–26. doi:10.1177/0961203311401457
68. Stockton KA, Kandiah DA, Paratz JD, Bennell KL. Fatigue, muscle strength and vitamin D status in women with systemic lupus erythematosus compared with healthy controls. *Lupus* (2012) **21**(3):271–8. doi:10.1177/0961203311425530
69. Schneider L, Colar da Silva AC, Werres Junior LC, Alegretti AP, Dos Santos AS, Santos M, et al. Vitamin D levels and cytokine profiles in patients with systemic lupus erythematosus. *Lupus* (2015) **24**(11):1191–7. doi:10.1177/0961203315584811
70. Ruiz-Irastorza G, Gordo S, Olivares N, Egurbide MV, Aguirre C. Changes in vitamin D levels in patients with systemic lupus erythematosus: effects on fatigue, disease activity, and damage. *Arthritis Care Res (Hoboken)* (2010) **62**(8):1160–5. doi:10.1002/acr.20186
71. Abou-Raya A, Abou-Raya S, Helmii M. The effect of vitamin D supplementation on inflammatory and hemostatic markers and disease activity in patients with systemic lupus erythematosus: a randomized placebo-controlled trial. *J Rheumatol* (2013) **40**(3):265–72. doi:10.3899/jrheum.111594
72. Andreoli L, Dall'Ara F, Piantoni S, Zanola A, Piva N, Cutolo M, et al. A 24-month prospective study on the efficacy and safety of two different monthly regimens of vitamin D supplementation in pre-menopausal women with systemic lupus erythematosus. *Lupus* (2015) **24**(4–5):499–506. doi:10.1177/0961203314559089
73. Aranow C, Kamen DL, Dall'Era M, Massarotti EM, Mackay MC, Koumpouras F, et al. Randomized, double-blind, placebo-controlled trial of the effect of vitamin D3 on the interferon signature in patients with systemic lupus erythematosus. *Arthritis Rheumatol* (2015) **67**(7):1848–57. doi:10.1002/art.39108
74. Piantoni S, Andreoli L, Scarsi M, Zanola A, Dall'Ara F, Pizzorni C, et al. Phenotype modifications of T-cells and their shift toward a Th2 response in patients with systemic lupus erythematosus supplemented with different monthly regimens of vitamin D. *Lupus* (2015) **24**(4–5):490–8. doi:10.1177/0961203314559090
75. Terrier B, Derian N, Schoindre Y, Chaara W, Geri G, Zahr N, et al. Restoration of regulatory and effector T cell balance and B cell homeostasis in systemic lupus erythematosus patients through vitamin D supplementation. *Arthritis Res Ther* (2012) **14**(5):R221. doi:10.1186/ar4060
76. Theodoratou E, Tzoulaki I, Zgaga L, Ioannidis JP. Vitamin D and multiple health outcomes: umbrella review of systematic reviews and meta-analyses of observational studies and randomised trials. *BMJ* (2014) **348**:g2035. doi:10.1136/bmj.g2035

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Systemic sclerosis patients present alterations in the expression of molecules involved in B-cell regulation

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The activation threshold of B cells is tightly regulated by an array of inhibitory and activator receptors in such a way that disturbances in their expression can lead to the appearance of autoimmunity. The aim of this study was to evaluate the expression of activating and inhibitory molecules involved in the modulation of B cell functions in transitional, naive, and memory B-cell subpopulations from systemic sclerosis patients. To achieve this, blood samples were drawn from 31 systemic sclerosis patients and 53 healthy individuals. Surface expression of CD86, MHC II, CD19, CD21, CD40, CD22, Siglec 10, CD35, and FcγRIIB was determined by flow cytometry. IL-10 production was evaluated by intracellular flow cytometry from isolated B cells. Soluble IL-6 and IL-10 levels were measured by ELISA from supernatants of stimulated B cells. Systemic sclerosis patients exhibit an increased frequency of transitional and naive B cells related to memory B cells compared with healthy controls. Transitional and naive B cells from patients express higher levels of CD86 and FcγRIIB than healthy donors. Also, B cells from patients show high expression of CD19 and CD40, whereas memory cells from systemic sclerosis patients show reduced expression of CD35. CD19 and CD35 expression levels associate with different autoantibody profiles. IL-10$^+$ B cells and secreted levels of IL-10 were markedly reduced in patients. In conclusion, systemic sclerosis patients show alterations in the expression of molecules involved in B-cell regulation. These abnormalities may be determinant in the B-cell hyperactivation observed in systemic sclerosis.

Keywords: regulatory B cells, systemic sclerosis, IL-10, FcγRIIb, Siglec

Introduction

Systemic sclerosis (SSc) is a systemic autoimmune disease characterized by an excessive deposition of extracellular matrix on skin and internal organs, vasculopathy, and the presence of a wide spectrum of autoantibodies. This disease is classified into limited cutaneous (lcSSc) and diffuse cutaneous (dcSSc) according to the degree of skin sclerosis, the presence of interstitial lung disease or pulmonary arterial hypertension, and the autoantibody profile (1). Fibrosis in SSc is produced by

a dysregulated reparation process, marked by the differentiation of tissue fibroblasts to myofibroblasts (2). When the gene expression pattern of SSc fibroblasts was compared with that of healthy individuals, no major differences were observed, which suggests that fibrosis could be caused by exogenous stimuli, such as those provided by the immune system (3). In that work, an increase in the expression of B cell-related genes together with an infiltration of $CD20^+$ B cells was observed in SSc skin, suggesting a pathogenic role of B cells.

Like most cells of the immune system, B cells express a wide array of activating and inhibitory receptors that modulate their activation status, allowing protective but controlled humoral immune responses. Abnormalities in the expression or function of these receptors on B cells have been reported in murine models of autoimmunity or in patients with autoimmune diseases (4–7). CD19 is a cell-surface signal transduction molecule that forms a complex with CD21, CD81, and CD225. CD21, or complement receptor 2 (CR2), binds to cleavage products of C3 complement component and conveys signals through CD19, thereby lowering the threshold for B-cell activation. CD19 also strengthens signals generated by the B-cell antigen receptor (BCR) and by CD40, which is activated by CD40 ligand (CD40L)-expressing T cells (8–10). Another complement receptor that is expressed on B cells – CD35 (CR1) – has been proposed to deliver inhibitory signals, thus opposing CD21 signals in the regulation of B-cell activation (11).

Fcγ receptor IIB (FcγRIIB) and members of sialic acid-binding immunoglobulin-type lectins (Siglecs) are other inhibitory receptors. FcγRIIB, a low-affinity receptor for the Fc fragment of immunoglobulin G (IgG), conveys inhibitory signals when cross-linked by IgG-containing immune complexes (12). Moreover, Siglecs are a family of immune receptors that recognize sialic acids attached to proteins. B cells express only two of them, CD22 (Siglec 2) and Siglec 10. Upon activation, CD22 recruits phosphatases that dephosphorylate several proteins, such as CD19, thus switching off B-cell activation. Although less studied, Siglec 10 presumably mediates inhibitory signals in a similar way to CD22 (4).

The multidimensional role of B cells in systemic autoimmune diseases has been progressively recognized (13). Increasing evidence suggests that B cells contribute to autoimmune responses by a series of antibody-independent mechanisms, which include antigen presentation to T cells and proinflammatory cytokine secretion (14). More recently, a population of IL-10-producing B cells with the ability to suppress autoimmune responses has been characterized in humans (15). These so-called regulatory B cells, which are enriched within the subpopulation of transitional B cells – immature B cells in transition to secondary lymphoid organs, have been shown to be numerically and/or functionally disturbed in patients with systemic autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (16, 17).

The present study was aimed at evaluating whether B-cell subpopulations from SSc patients, including transitional B cells, present alterations in frequency, phenotype, and/or expression of activating and inhibitory receptors compared with those from healthy subjects.

Materials and Methods

Study Subjects

Thirty-one patients meeting the American College of Rheumatology criteria for SSc (18) and 53 healthy controls were recruited. The involvement of different systems or organs was evaluated with the Modified Medsger scale (19). **Table 1** shows the main clinical and demographic characteristics of both groups. Blood samples (50 ml) were drawn by venous puncture for B-cell phenotyping. Due to the limited amount of sample, only some parameters could be assessed for each individual. For the expression levels of CD86, CD40, major histocompatibility class II (MHC II) molecules, CD35, CD21, CD22, and Siglec 10 on B cells, sex- and age-matched SSc patients and healthy controls groups were compared. The study was approved by the Ethical Committees of the Hospital Clínico and Facultad de Medicina, Universidad de Chile, and all subjects gave written informed consent in accordance with the Declaration of Helsinki.

B-Cell Phenotyping

We characterized B cells using the following monoclonal anti-human antibodies: anti-CD19 FITC, CD19 Alexa Fluor 700, CD24 PE-Cy7, CD38 APC, CD27 APC, CD86 PE-Cy5, CD25

TABLE 1 | Main demographic and clinical characteristics of the systemic sclerosis patients and healthy controls recruited for this study.

Characteristics	Patients ($n = 31$)	Controls ($n = 53$)
Female/male	23/8	28/25
Age	49.3 ± 11.8	40.0 ± 13.7
Disease duration, months (mean ± SD)	102.2 ± 107.2	
lcSSc/dcSSc	22/9	
Rodnan score (mean ± SD)	13.9 ± 6.0	
ANA positivity, *n* (%)	31 (100)	
ANA pattern,[a] *n* (%)		
Speckled	10 (32.3)	
Nucleolar	8 (25.8)	
Homogeneous	9 (29.0)	
Centromere	14 (45.2)	
Anti-Scl-70 positivity, *n* (%)	6 (19.4)	
Organ involvement,[b] *n* (%)		
Peripheral vascular	16 (51.6)	
Skin	29 (93.5)	
Gastrointestinal tract	27 (90.0)	
Lung	21 (70)	
Heart	16 (51.6)	
Kidney	4 (12.9)	
Therapy		
Prednisone	3/31	
Azathioprine + prednisone	2/31	
Methotrexate	3/31	
D-penicillamine	1/31	
Methotrexate + D-penicillamine	1/31	
Methotrexate + D-penicillamine + prednisone	1/31	
Hydroxychloroquine	4/31	
Methotrexate + hydroxychloroquine	1/31	
Only symptomatic treatment	15/31	

SD, standard deviation; lcSSc, limited cutaneous systemic sclerosis; dcSSc, diffuse cutaneous systemic sclerosis; ANA, antinuclear antibodies.

[a]*Some patients have more than one pattern.*

[b]*Defined as a Modified Medsger scale value ≥1.*

PE-Cy7, CD1d PE, CD21 PE, CD22 PE, CD35 PE, Siglec 10 PE (Biolegend, USA), CD40 FITC, IL-10 PE, MHC II APC eFluor 780 (eBioscience, USA), and FcγRII PE (clone 7.3; Fitzgerald Industries International, USA). For the cell surface staining procedure, peripheral blood mononuclear cells (PBMCs) were obtained from blood samples by density gradient centrifugation with Lymphoprep (Stemcell Technologies, Canada) and either stained freshly or cryopreserved in liquid nitrogen until use. Cells were incubated with fluorochrome-labeled antibodies for 30 min at 4°C, washed, and fixed before acquisition on a FACSCalibur or FACSAria III flow cytometer (BD Biosciences). Data were analyzed with FloJo 7.6 Software (USA).

For cytokine production assays, untouched B cells were isolated from whole blood (EasySep, Stemcell Technologies) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone, Thermo Scientific, USA) at 1×10^6 cells/ml in 96-well plates with 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 1 μg/ml ionomycin (Sigma-Aldrich, USA), and 1 μg/ml brefeldin A (eBioscience) for 5 h at 37°C and 5% CO_2. For intracellular detection of IL-10 production on B-cell subpopulations, cells were stained with anti-CD19, anti-CD38, and anti-CD24 antibodies, fixed and permeabilized with Permeabilization Buffer (eBioscience), and incubated with an anti-IL-10 antibody for flow cytometry analysis. Fluorescence minus one (FMO) staining controls were used to exclude non-specific background staining. Culture supernatants were collected for detection of IL-10 and IL-6 levels by ELISA (eBioscience).

Statistical Analyses

All the study variables were tested for normal distribution with the D'Agostino-Pearson omnibus test. Differences between SSc patients and healthy control groups, or between groups of patients, were analyzed using the two-tailed unpaired Student's *t*-test or Mann–Whitney *U* test, when appropriate. For matched groups, the two-tailed paired Student's *t*-test or Wilcoxon signed-rank test were used, when appropriate. To examine the relationship between continuous variables, linear regression analyses were performed. For statistic analyses and graphics, Stata 12 and GraphPad Prism 5 softwares were used.

Results

Altered Frequencies of B-Cell Subpopulations in Peripheral Blood of Systemic Sclerosis Patients

To investigate whether the frequency of different B-cell subpopulations is altered in peripheral blood of SSc patients, we analyzed them by flow cytometry. A region was set to define the lymphocytic population according to forward and side scatter patterns. B cells were defined as $CD19^+$ cells, and a second region was set for them. Finally, CD24 and CD38 expression was used to discriminate transitional ($CD24^{high}CD38^{high}$) from naive ($CD24^{int}CD38^{int}$) and memory ($CD24^{high}CD38^{-}$) B cells, as previously described (16) (**Figure 1A**).

An increased percentage of $CD19^+$ B cells was found in PBMC of SSc patients compared with healthy controls (**Figure 1B**). Since the relative frequency of memory B cells was dramatically decreased within SSc patients' B cells, the observed

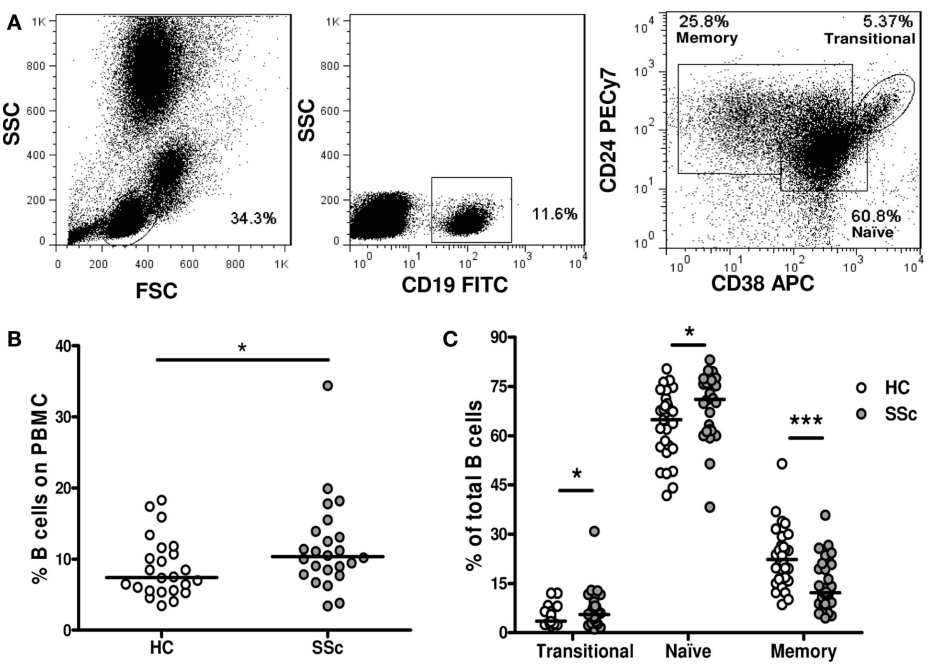

FIGURE 1 | Frequencies of B-cell subpopulations in systemic sclerosis patients. **(A)** Flow cytometry gating strategy to identify transitional B cells ($CD19^+CD24^{high}CD38^{high}$), naive B cells ($CD19^+CD24^{int}CD38^{int}$), and memory B cells ($CD19^+CD24^{high}CD38^-$). **(B)** Percentage of $CD19^+$ B cells within peripheral blood mononuclear cells (PBMC) in healthy controls (HC) ($n = 24$) and systemic sclerosis (SSc) patients ($n = 24$). **(C)** Percentage of transitional, naive, or memory B-cell subpopulations within total $CD19^+$ B cells in HC (white circles) ($n = 31$) and SSc patients (gray circles) ($n = 30$). $^*P < 0.05$, $^{***}P < 0.001$, Mann–Whitney *U* test.

increase in the percentage of total B cells can be explained by an expansion of naive B cells. Interestingly, the percentage of transitional B cells among total B cells was also increased in the peripheral blood of SSc patients compared with healthy subjects (**Figure 1C**).

B Cells from Systemic Sclerosis Patients Exhibit an Activated Phenotype

To evaluate whether B cells from SSc patients exhibit an activated phenotype, the surface expression of MHC II and CD86 molecules, involved in antigen presentation and costimulation, respectively, and upregulated upon B-cell activation, was measured (**Figure 2**). Although very low, the expression of CD86 was elevated in B cells from SSc patients, particularly in the transitional and naive B-cell subpopulations, when compared with healthy subjects (**Figure 2B**). In contrast, no differences were observed in MHC II expression (**Figure 2C**).

IL-6 and IL-10 are two B-cell-secreted cytokines that have been involved in the SSc fibrotic process (2). These cytokines were assessed in isolated and stimulated B cells as an estimation of their activation status. No differences were observed in the levels of IL-6 secreted by B cells from SSc patients and healthy controls (**Figure 3A**). However, SSc patients exhibited a significantly lower IL-10 secretion in comparison to healthy subjects (**Figure 3B**). To explore which B-cell subpopulation was responsible for this decrease in IL-10 production, intracellular IL-10 expression was evaluated by flow cytometry. The majority of IL-10^{+} cells was found within transitional B cells, both in SSc patients and healthy controls, which is in accordance with previous reports (**Figure 3C**) (16). Of note, the percentage of IL-10^{+} B cells was reduced in all B-cell subpopulations of SSc patients (**Figure 3D**). To further characterize this finding, we studied in a subset of seven patients and eight healthy subjects the frequency of CD25^{high}CD27^{high}CD86^{high}CD1d^{high} B cells, since this population has been described to express high levels of IL-10 (and also TGF-β) and to exhibit strong regulatory properties (20). In accordance with the decreased frequencies of IL-10^{+} B cells, SSc patients presented reduced percentages of CD25^{high}CD27^{high}CD86^{high}CD1d^{high} B cells compared with healthy controls (**Figure 3E**).

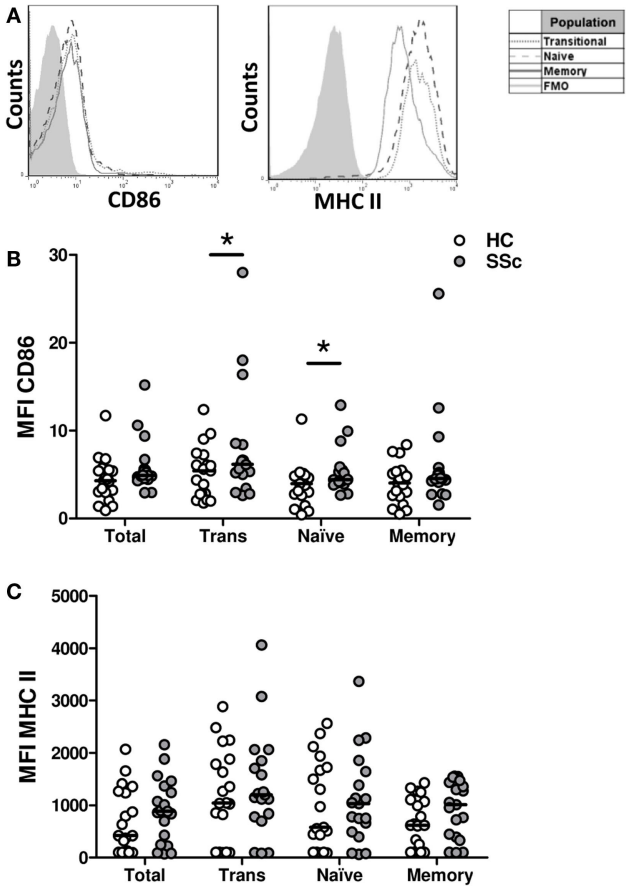

FIGURE 2 | Surface expression of CD86 and major histocompatibility class II (MHC II) molecules on B cells from systemic sclerosis patients. **(A)** Representative histograms of the expression of CD86 and MHC II on transitional (dotted line), naive (dashed line), or memory B cells (solid line). The shaded curve represents the fluorescence minus one (FMO) control staining. **(B,C)** Expression of CD86 **(B)** and MHC II **(C)** on total CD19^{+} B cells, transitional B cells (Trans), naive B cells and memory B cells in healthy controls (HC, white circles) (n = 19) and systemic sclerosis patients (SSc, gray circles) (n = 19). *P < 0.05, Wilcoxon signed-rank test. MFI, mean fluorescence intensity.

B Cells from Systemic Sclerosis Patients Exhibit a Shift in the Balance of Activating and Inhibitory Receptors

The activated phenotype displayed by B cells from SSc patients could be caused by an overexpression of molecules involved in B-cell activation. To test this possibility, the expression of three molecules that participate in B-cell activation was measured by flow cytometry: CD19, CD21, and CD40. In accordance with previous reports (21), B cells from SSc patients displayed a high expression of CD19 not only in naive and memory subpopulations but also in the transitional subset (**Figure 4A**). The expression of CD19 on total B cells and on each subpopulation was even higher in a subset of SSc patients with anti-Scl-70 antibodies, which is mainly associated with dcSSc (**Figure 4B**). In contrast, no differences were observed when comparing CD21 expression on different B-cell subpopulations from SSc patients and healthy controls (**Figure 4C**). Regarding CD40, increased expression levels were observed in total B cells as well as in all B-cell subpopulations from SSc patients (**Figure 4D**).

To find out if a defective expression of inhibitory receptors could be associated with the B-cell hyperactivity observed in SSc patients, the surface expression of CD22, Siglec 10, CD35, and FcγRIIB was examined on B cells from SSc patients and healthy subjects. Neither CD22 nor Siglec 10 showed altered expression levels in any of the B-cell subpopulations from SSc patients that were studied (**Figures 5A,B**). In contrast, SSc patients exhibited lower levels of CD35 expression in CD19^{+} B cells, in particular in the memory compartment (**Figure 5C**). Moreover, the subset of patients with anticentromere antibodies, which associate with lcSSc, showed an increased expression of CD35 in total B cells and in all subpopulations (**Figure 5D**). Unexpectedly, FcγRIIB expression was found to be significantly increased on naive and transitional B-cell subsets, but not on memory B cells, from SSc patients (**Figure 5E**).

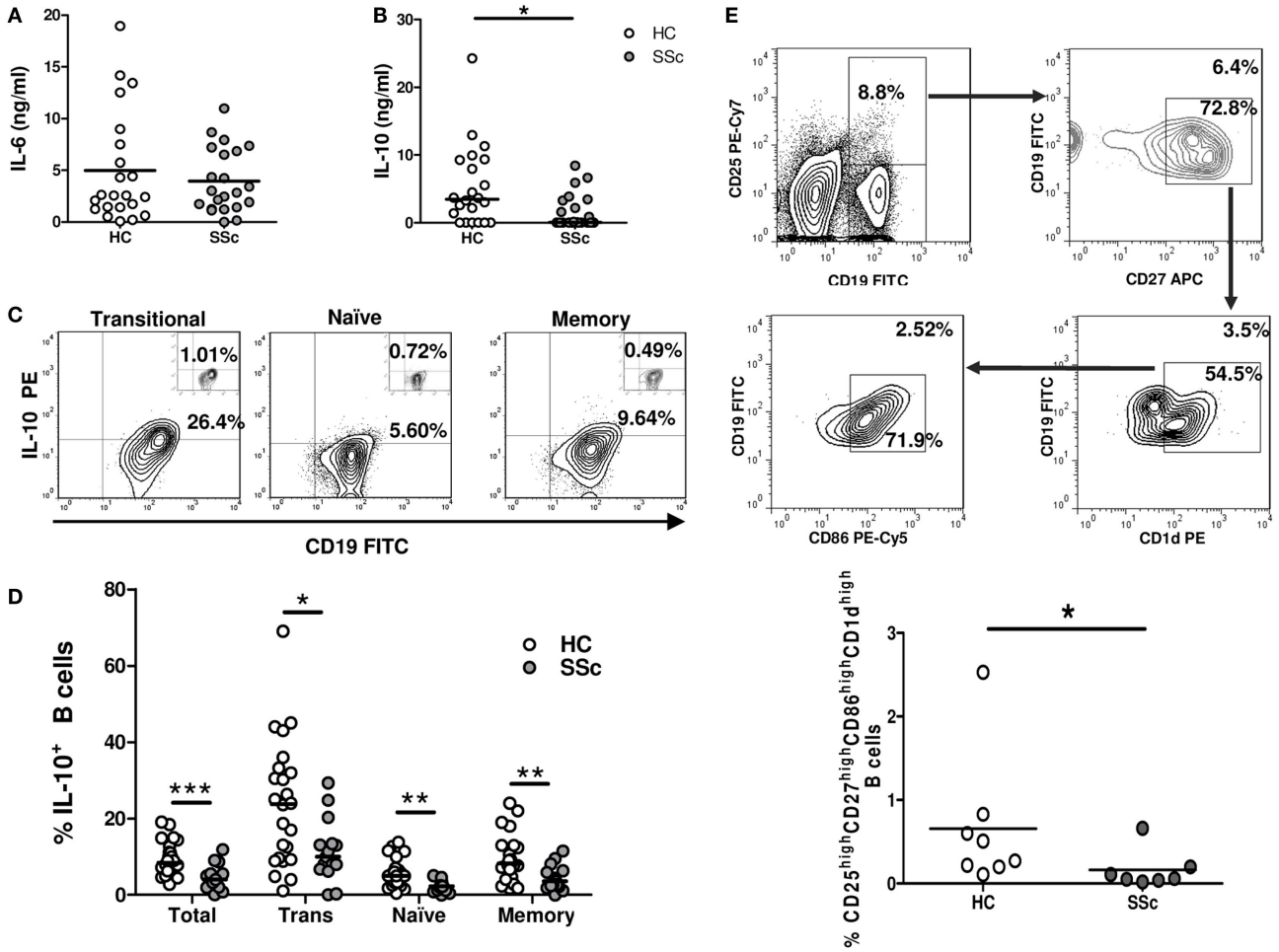

FIGURE 3 | Reduced IL-10-expressing B-cell frequencies in systemic sclerosis patients. **(A–D)** Isolated B cells from healthy controls (HC) or systemic sclerosis patients (SSc) were stimulated for 5 h with PMA and ionomycin. IL-6 levels **(A)** and IL-10 levels **(B)** secreted by B cells from HC ($n = 22$) or SSc ($n = 22$) were determined by ELISA and compared with the Mann–Whitney *U* test. **(C)** Representative plots of the percentage of $CD19^{+}IL\text{-}10^{+}$ B cells within transitional (left), naive (middle), and memory (right) populations. The small inserts on each plot represent the background percentages, as determined by the fluorescence minus one (FMO) control staining. **(D)** Graph summarizing the percentages of $CD19^{+}IL\text{-}10^{+}$ cells among total, transitional (Trans), naive, and memory B cells in HC (white circles) ($n = 24$) and SSc (gray circles) ($n = 15$) groups. Statistic comparisons were made using the unpaired Student's *t*-test. **(E)** Peripheral blood mononuclear cells from HC ($n = 8$) and SSc patients ($n = 7$) were stained and analyzed for the presence of $CD25^{high}CD27^{high}CD86^{high}CD1d^{high}$ B cells and compared with the Mann–Whitney *U* test. The upper panel represents the gating strategy to identify the subpopulation. Numbers outside and inside the gates indicate the percentages of gated cells from the total or previously gated B cells, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Of interest, patients exhibiting peripheral vascular alterations, as assessed by the Modified Medsger scale, showed a low expression of CD22 and CD35 in total B cells and in all B-cell subpopulations (**Figure 6**), suggesting a critical role of inhibitory molecules on B cells in the vascular component of this disease.

Discussion

In the present study, we found important alterations in the frequency of different B-cell subpopulations and in the balance between activating and inhibitory molecules expressed by these B-cell subsets in SSc patients. SSc patients exhibit a bias in the distribution of B-cell subpopulations toward an increase in the relative frequency of naive cells and a reduction of the memory compartment. These results, as well as the increase in the percentage of $CD19^{+}$ B cells within PBMC, are in agreement with a previous study in Japanese patients (21). However, this study describes for the first time an increase in the proportion of the transitional B-cell subpopulation in SSc patients. Similar findings have been shown for other autoimmune diseases, such as SLE and primary Sjögren's syndrome (22).

Although it has been suggested that an expansion of immature forms of B cells in these conditions could be caused by failures in early tolerance checkpoints (23), some of these autoreactive transitional B cells could correspond to IL-10-secreting regulatory B cells. Furthermore, we found a reduced percentage of $IL\text{-}10^{+}$ B cells in the peripheral blood of SSc patients, not only in the transitional subpopulation but also in memory and naive B

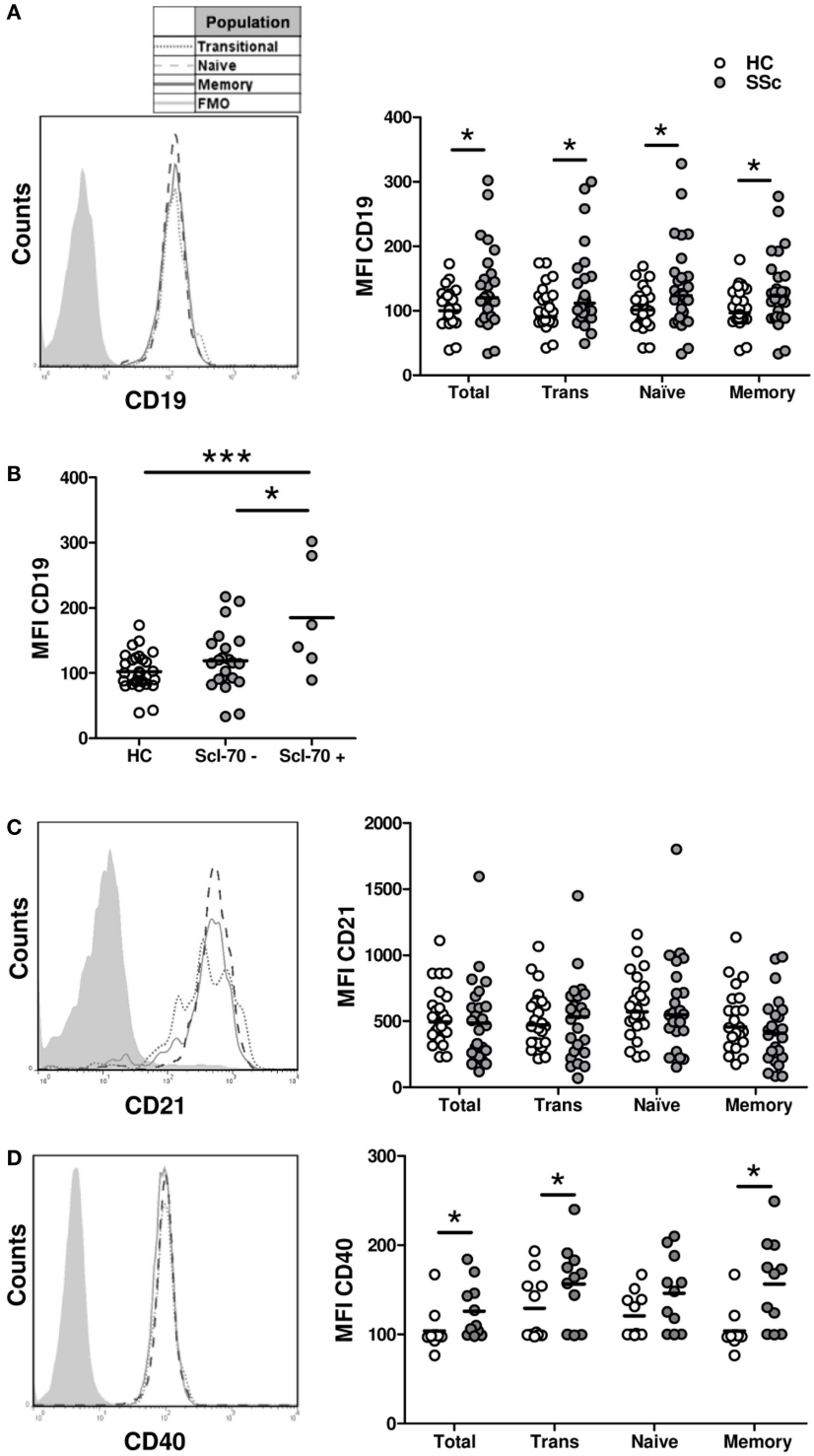

FIGURE 4 | Expression of the activation molecules CD19 (A,B), CD21 (C), and CD40 (D) in B cells from systemic sclerosis patients (n = 29, 24, and 19, respectively) and healthy subjects (n = 28, 24, and 19, respectively). **(A,C,D)** Left: representative histograms of the expression of each molecule on transitional (dotted line), naive (dashed line), or memory B cells (solid line). The shaded curve represents the fluorescence minus one (FMO) control staining. Right: graphs summarizing the expression of each molecule on total CD19$^+$ B cells, transitional B cells (Trans), naive B cells, and memory B cells in healthy controls (HC, white circles) and systemic sclerosis patients (SSc, gray circles). **(B)** Expression of CD19 in total B cells from HC and SSc patients classified according to the presence of anti-Scl-70 antibodies. *P < 0.05, **P < 0.01, ***P < 0.01. Mann–Whitney U test for graphs in **(A,C)**, Wilcoxon signed-rank test for graphs in **(D)**, and unpaired Student's t-test for graph in **(B)**. MFI, mean fluorescence intensity.

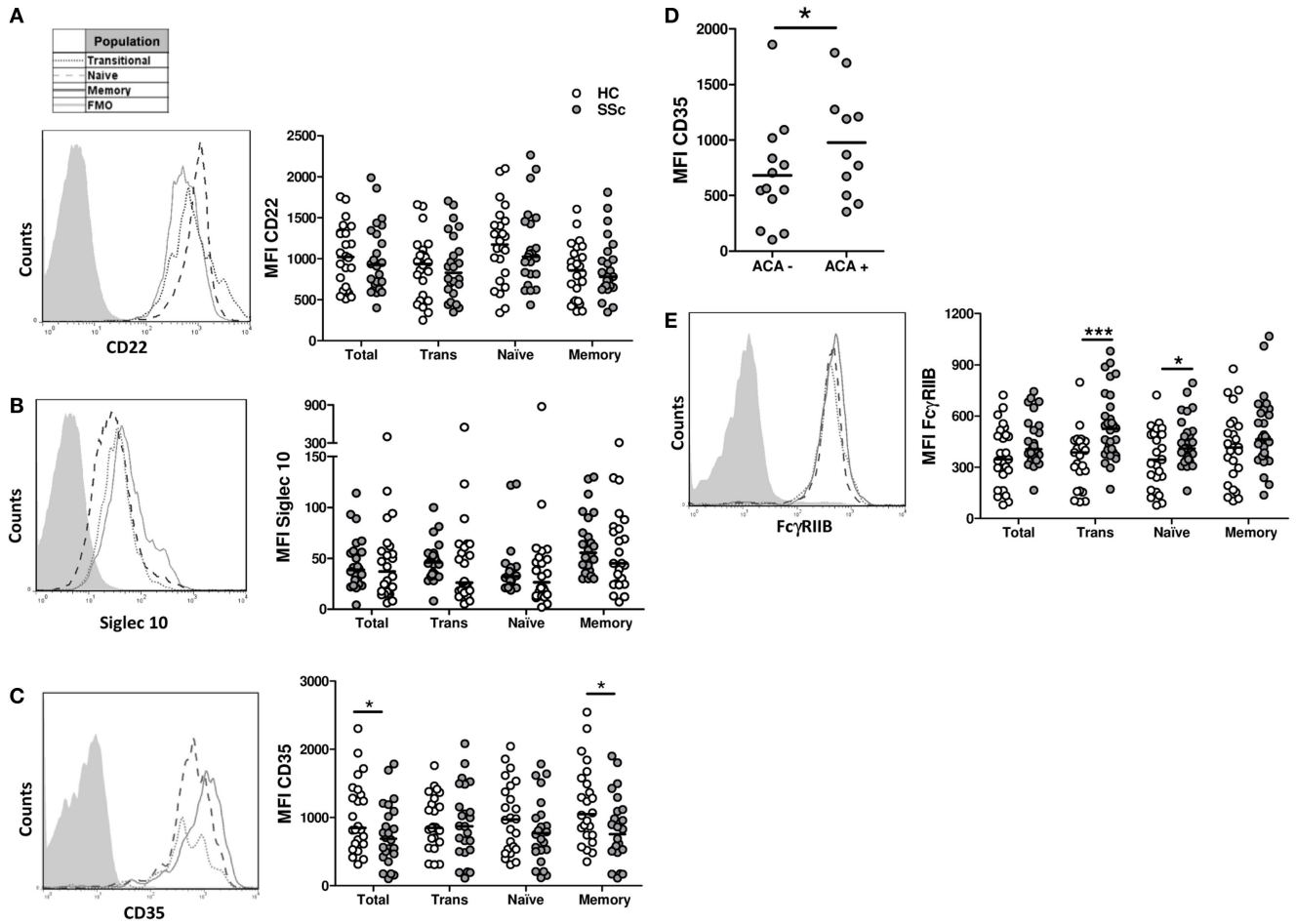

FIGURE 5 | Expression of the inhibition receptors CD22 (A), Siglec 10 (B), CD35 (C,D), and FcγRIIB (E) in B cells from systemic sclerosis patients (n = 24, 24, 24, and 30, respectively) and healthy subjects (n = 24, 24, 24, and 25, respectively). **(A–C,E)** Left: representative histograms of the expression of each molecule on transitional (dotted line), naive (dashed line), or memory B cells (solid line). The shaded curve represents the fluorescence minus one (FMO) control staining. Right: graphs summarizing the expression of each molecule on total CD19+ B cells, transitional B cells (Trans), naive B cells, and memory B cells in healthy controls (HC, white circles) and systemic sclerosis patients (SSc, gray circles). **(D)** Expression of CD35 in SSc patients according to the presence of anticentromere antibodies (ACA). $^{*}P < 0.05$, $^{***}P < 0.001$. Paired Student's t-test for graphs in **(A,C)**, Wilcoxon signed-rank test for graphs in **(B)**, and unpaired Student's t-test for graphs in **(D,E)**. MFI, mean fluorescence intensity.

cells. Moreover, SSc patients also exhibited reduced percentages of $CD25^{high}CD27^{high}CD86^{high}CD1d^{high}$ B cells, a B-cell subpopulation able to suppress $CD4^{+}$ T-cell proliferation (20). Alterations in regulatory B-cell functions have been observed in other autoimmune diseases (16, 17, 24), and SSc may not be an exception; however, functional analysis, such as T-cell responses-inhibition assays, should be performed before drawing such conclusion.

B cells from SSc patients have been reported to present an activated phenotype, which leads to an overproduction of Ig, including autoantibodies, and an active production of cytokines, such as profibrotic IL-6 (25). In the present study, we demonstrated that naive and transitional B cells from SSc patients exhibit an activated phenotype, revealed by an increased expression of CD86 compared with healthy controls. In contrast with previously reported results, this difference was not detected in memory B cells (21). Resting naive B cells have been described to cause an incomplete activation of T cells, in part, due to the lack of expression of CD86, which can be restored after stimulation (26, 27). Therefore, an upregulation of CD86 on activated naive B cells could contribute to SSc pathogenesis by activating autoreactive T cells, which in turn stimulate the secretion of profibrotic cytokines by fibroblasts (28). In contrast, regulatory B cells expressing markers of transitional B cells are active promoters of tolerance, as they are able to induce regulatory T cells, a function that is largely dependent on CD86 (24). The impact of the increased CD86 expression found in transitional B cells from SSc patients requires further investigation.

The activated phenotype of B cells in SSc has been attributed to an increased expression of the activating molecules CD19 and CD21 (29). In addition, a polymorphism in the CD22 coding gene that is associated with a decreased expression of this receptor on B cells has been reported to be more frequent in a subset of Japanese lcSSc patients (30). Similarly, in the *tight skin* TSK/+ murine model of SSc, hyperresponsive B cells depend on an exacerbated activity of CD19 and an impaired counterregulation by CD22 (31, 32). In

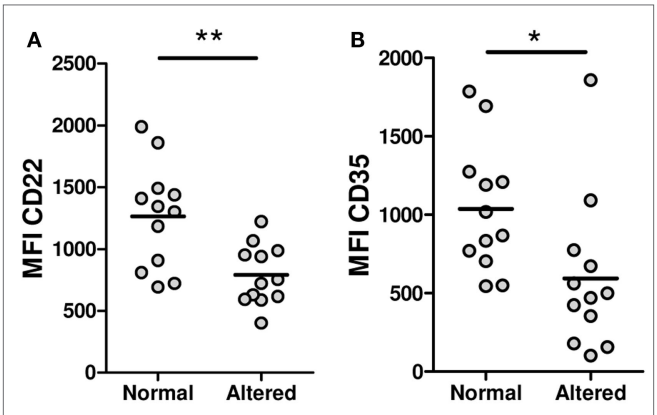

FIGURE 6 | Expression levels of CD22 (A) and CD35 (B) on total B cells from systemic sclerosis patients with a peripheral vascular Modified Medsger scale value = 0 (normal) or ≥1 (altered). *$P < 0.05$, **$P < 0.01$, unpaired Student's *t*-test. MFI, mean fluorescence intensity.

the results presented herein, an increased expression of CD19 and CD40, but not of CD21, was found in SSc B cells. The differences observed between this study and previous ones, regarding the expression of B-cell surface molecules such as CD40, CD21, and CD86 or the secretion of IL-10 and IL-6 by SSc B cells, could be attributed to different experimental settings or to the composition of the study group, in terms of the proportion of patients presenting lcSSc or dcSSc, as well as the undergoing therapy (21, 29). Indeed, decreased expression of CD40 and augmented levels of CD22 were found in patients receiving steroids (data not shown).

On the other hand, although no differences were found in the expression of CD22 or Siglec 10 on B cells from SSc patients or healthy subjects, a reduced expression of CD35 was detected in memory B cells from SSc patients. A reduced expression of this inhibitory complement receptor has been previously reported for other autoimmune diseases, such as SLE and RA, but never before for SSc (33–35). On human B cells, CD35 inhibits the BCR- and CD40-induced increase in cytoplasmic Ca^{2+} levels, proliferation, and antibody secretion (11, 33). This suggests a role of CD35 as a late checkpoint in preventing the maturation and differentiation of autoreactive B cells, a function that could be altered in autoimmune diseases such as SSc (6). In this sense, it is remarkable to note that in this group of patients, expression levels of molecules involved in the regulation of B-cell activity are associated with different subsets of patients. For instance, patients carrying anti-Scl-70 antibodies, which are characteristic of dcSSc, exhibited high expression levels of CD19 while patients carrying anticentromere antibodies, which are related to lcSSc, showed increased expression levels of CD35. The immunologic basis of these findings remains obscure for us.

Intriguingly, naive and transitional B cells from SSc patients showed high expression levels of the inhibitory receptor FcγRIIB. Our group and others have described that in autoimmune diseases, such as RA and SLE, B cells exhibit a reduced expression of this receptor in the memory subpopulation, associated to high levels of autoantibodies (34–38). Moreover, the absence of FcγRIIB specifically on B cells predisposes for the development of lupus and arthritis in animal models, highlighting its role as a crucial molecule for the control of autoimmune humoral responses (39). Although circulating human transitional B cells have been initially described to express moderate levels of FcγRIIB, the function of this receptor in transitional B cells remains unknown (22). A more recent publication reports a subpopulation of mouse IL-10-secreting B cells that expresses high levels of FcγRIIB, which endows these cells with the ability for an efficient endocytosis of immune complexes and inhibition of $CD4^+$ T-cell responses. The authors postulate that by capturing immune complexes, these $FcγRIIB^{high}$ B cells may attenuate the activation of immune responses (40). It remains unclear how the increased expression of FcγRIIB on the transitional subpopulation could be involved in the alterations observed in SSc B cells. One possibility is that it may be a compensatory mechanism triggered after the development of an exacerbated humoral response. Another explanation is that FcγRIIB could inhibit IL-10 secretion by transitional B cells after binding autoantibody-containing immune complexes, thus precluding its regulatory functions.

Altogether, this study demonstrates alterations in the frequencies and activation status of different B-cell subpopulations from SSc patients, including for the first time an analysis of the transitional B-cell subpopulation. We are currently carrying out *in vitro* experiments in order to uncover the mechanisms through which these abnormalities affect the function of B cells in this disease as well as key signaling molecules involved.

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References

1. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. *N Engl J Med* (2009) **360**:1989–2003. doi:10.1056/NEJMra0806188
2. Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. *J Clin Invest* (2007) **117**:557–67. doi:10.1172/JCI31139
3. Whitfield ML, Finlay DR, Murray JI, Troyanskaya OG, Chi JT, Pergamenschikov A, et al. Systemic and cell type-specific gene expression patterns in scleroderma skin. *Proc Natl Acad Sci U S A* (2003) **100**:12319–24. doi:10.1073/pnas.1635114100
4. Muller J, Nitschke L. The role of CD22 and Siglec-G in B-cell tolerance and autoimmune disease. *Nat Rev Rheumatol* (2014) **10**:422–8. doi:10.1038/nrrheum.2014.54
5. Smith KG, Clatworthy MR. FcgammaRIIB in autoimmunity and infection: evolutionary and therapeutic implications. *Nat Rev Immunol* (2010) **10**:328–43. doi:10.1038/nri2762
6. Erdei A, Isaak A, Torok K, Sándor N, Kremlitzka M, Prechl J, et al. Expression and role of CR1 and CR2 on B and T lymphocytes under physiological and autoimmune conditions. *Mol Immunol* (2009) **46**:2767–73. doi:10.1016/j.molimm.2009.05.181

7. Dorner T, Lipsky PE. Signalling pathways in B cells: implications for autoimmunity. *Curr Top Microbiol Immunol* (2006) **305**:213–40. doi:10.1007/3-540-29714-6_11
8. Fujimoto M, Poe JC, Hasegawa M, Tedder TF. CD19 regulates intrinsic B lymphocyte signal transduction and activation through a novel mechanism of processive amplification. *Immunol Res* (2000) **22**:281–98. doi:10.1385/IR:22:2-3:281
9. Mongini PK, Vilensky MA, Highet PF, Inman JK. The affinity threshold for human B cell activation via the antigen receptor complex is reduced upon co-ligation of the antigen receptor with CD21 (CR2). *J Immunol* (1997) **159**:3782–91.
10. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev* (2009) **229**:152–72. doi:10.1111/j.1600-065X.2009.00782.x
11. Jozsi M, Prechl J, Bajtay Z, Erdei A. Complement receptor type 1 (CD35) mediates inhibitory signals in human B lymphocytes. *J Immunol* (2002) **168**:2782–8. doi:10.4049/jimmunol.168.6.2782
12. Nimmerjahn F, Ravetch JV. Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol* (2008) **8**:34–47. doi:10.1038/nri2206
13. Mauri C, Bosma A. Immune regulatory function of B cells. *Annu Rev Immunol* (2012) **30**:221–41. doi:10.1146/annurev-immunol-020711-074934
14. Fillatreau S. Cytokine-producing B cells as regulators of pathogenic and protective immune responses. *Ann Rheum Dis* (2013) **72**(Suppl 2):ii80–4. doi:10.1136/annrheumdis-2012-202253
15. Berthelot JM, Jamin C, Amrouche K, Le Goff B, Maugars Y, Youinou P. Regulatory B cells play a key role in immune system balance. *Joint Bone Spine* (2013) **80**:18–22. doi:10.1016/j.jbspin.2012.04.010
16. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic lupus erythematosus patients. *Immunity* (2010) **32**:129–40. doi:10.1016/j.immuni.2009.11.009
17. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, et al. CD19 + CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. *Sci Transl Med* (2013) **5**:173ra23. doi:10.1126/scitranslmed.3005407
18. Masi AT. Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* (1980) **23**:581–90. doi:10.1002/art.1780230510
19. Medsger TA Jr, Bombardieri S, Czirjak L, Scorza R, Della Rossa A, Bencivelli W. Assessment of disease severity and prognosis. *Clin Exp Rheumatol* (2003) **21**:S42–6.
20. Kessel A, Haj T, Peri R, Snir A, Melamed D, Sabo E, et al. Human CD19(+) CD25(high) B regulatory cells suppress proliferation of CD4(+) T cells and enhance Foxp3 and CTLA-4 expression in T-regulatory cells. *Autoimmun Rev* (2012) **11**:670–7. doi:10.1016/j.autrev.2011.11.018
21. Sato S, Fujimoto M, Hasegawa M, Takehara K. Altered blood B lymphocyte homeostasis in systemic sclerosis: expanded naive B cells and diminished but activated memory B cells. *Arthritis Rheum* (2004) **50**:1918–27. doi:10.1002/art.20274
22. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. *Blood* (2005) **105**:4390–8. doi:10.1182/blood-2004-11-4284
23. Landolt-Marticorena C, Wither R, Reich H, Herzenberg A, Scholey J, Gladman DD, et al. Increased expression of B cell activation factor supports the abnormal expansion of transitional B cells in systemic lupus erythematosus. *J Rheumatol* (2011) **38**:642–51. doi:10.3899/jrheum.100214
24. Lemoine S, Morva A, Youinou P, Jamin C. Human T cells induce their own regulation through activation of B cells. *J Autoimmun* (2011) **36**:228–38. doi:10.1016/j.jaut.2011.01.005
25. Matsushita T, Hasegawa M, Yanaba K, Kodera M, Takehara K, Sato S. Elevated serum BAFF levels in patients with systemic sclerosis: enhanced BAFF signaling in systemic sclerosis B lymphocytes. *Arthritis Rheum* (2006) **54**:192–201. doi:10.1002/art.21526
26. Jiang W, Lederman MM, Harding CV, Sieg SF. Presentation of soluble antigens to CD8 + T cells by CpG oligodeoxynucleotide-primed human naive B cells. *J Immunol* (2011) **186**:2080–6. doi:10.4049/jimmunol.1001869
27. Good KL, Avery DT, Tangye SG. Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naive B cells. *J Immunol* (2009) **182**:890–901. doi:10.4049/jimmunol.182.2.890
28. O'Reilly S, Hugle T, van Laar JM. T cells in systemic sclerosis: a reappraisal. *Rheumatology (Oxford)* (2012) **51**:1540–9. doi:10.1093/rheumatology/kes090
29. Sato S, Hasegawa M, Fujimoto M, Tedder TF, Takehara K. Quantitative genetic variation in CD19 expression correlates with autoimmunity. *J Immunol* (2000) **165**:6635–43. doi:10.4049/jimmunol.165.11.6635
30. Hitomi Y, Tsuchiya N, Hasegawa M, Fujimoto M, Takehara K, Tokunaga K, et al. Association of CD22 gene polymorphism with susceptibility to limited cutaneous systemic sclerosis. *Tissue Antigens* (2007) **69**:242–9. doi:10.1111/j.1399-0039.2007.00801.x
31. Saito E, Fujimoto M, Hasegawa M, Fujimoto M, Takehara K, Tokunaga K, et al. CD19-dependent B lymphocyte signaling thresholds influence skin fibrosis and autoimmunity in the tight-skin mouse. *J Clin Invest* (2002) **109**:1453–62. doi:10.1172/JCI0215078
32. Asano N, Fujimoto M, Yazawa N, Shirasawa S, Hasegawa M, Okochi H, et al. B lymphocyte signaling established by the CD19/CD22 loop regulates autoimmunity in the tight-skin mouse. *Am J Pathol* (2004) **165**:641–50. doi:10.1016/S0002-9440(10)63328-7
33. Kremlitzka M, Polgar A, Fulop L, Kiss E, Poór G, Erdei A. Complement receptor type 1 (CR1, CD35) is a potent inhibitor of B-cell functions in rheumatoid arthritis patients. *Int Immunol* (2013) **25**:25–33. doi:10.1093/intimm/dxs090
34. Prokopec KE, Rhodiner M, Matt P, Lindqvist U, Kleinau S. Down regulation of Fc and complement receptors on B cells in rheumatoid arthritis. *Clin Immunol* (2010) **137**:322–9. doi:10.1016/j.clim.2010.08.006
35. Isaak A, Gergely P Jr, Szekeres Z, Prechl J, Poór G, Erdei A, et al. Physiological up-regulation of inhibitory receptors Fc gamma RII and CR1 on memory B cells is lacking in SLE patients. *Int Immunol* (2008) **20**:185–92. doi:10.1093/intimm/dxm132
36. Catalan D, Aravena O, Sabugo F, Wurmann P, Soto L, Kalergis AM, et al. B cells from rheumatoid arthritis patients show important alterations in the expression of CD86 and FcgammaRIIb, which are modulated by anti-tumor necrosis factor therapy. *Arthritis Res Ther* (2010) **12**:R68. doi:10.1186/ar2985
37. Su K, Yang H, Li X, Li X, Gibson AW, Cafardi JM, et al. Expression profile of FcgammaRIIb on leukocytes and its dysregulation in systemic lupus erythematosus. *J Immunol* (2007) **178**:3272–80. doi:10.4049/jimmunol.178.5.3272
38. Mackay M, Stanevsky A, Wang T, Aranow C, Li M, Koenig S, et al. Selective dysregulation of the FcgammaIIB receptor on memory B cells in SLE. *J Exp Med* (2006) **203**:2157–64. doi:10.1084/jem.20051503
39. Brownlie RJ, Lawlor KE, Niederer HA, Cutler AJ, Xiang Z, Clatworthy MR, et al. Distinct cell-specific control of autoimmunity and infection by FcgammaRIIb. *J Exp Med* (2008) **205**:883–95. doi:10.1084/jem.20072565
40. Qian L, Qian C, Chen Y, Bai Y, Bao Y, Lu L, et al. Regulatory dendritic cells program B cells to differentiate into CD19hiFcgammaIIbhi regulatory B cells through IFN-beta and CD40L. *Blood* (2012) **120**:581–91. doi:10.1182/blood-2011-08-377242

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Breast milk and solid food shaping intestinal immunity

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After birth, the intestinal immune system enters a critical developmental stage, in which tolerogenic and pro-inflammatory cells emerge to contribute to the overall health of the host. The neonatal health is continuously challenged by microbial colonization and food intake, first in the form of breast milk or formula and later in the form of solid food. The microbiota and dietary compounds shape the newborn immune system, which acquires the ability to induce tolerance against innocuous antigens or induce pro-inflammatory immune responses against pathogens. Disruption of these homeostatic mechanisms might lead to undesired immune reactions, such as food allergies and inflammatory bowel disease. Hence, a proper education and maturation of the intestinal immune system is likely important to maintain life-long intestinal homeostasis. In this review, the most recent literature regarding the effects of dietary compounds in the development of the intestinal immune system are discussed.

Keywords: oral tolerance, breast milk, dietary compounds, microbiota, intestinal immunity

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Introduction

The gastrointestinal system is one of the largest vulnerable surfaces of our body. It is continuously facing the external environment, including microbiota, nutrients, metabolites, pollutants, and harmful pathogens. To maintain intestinal homeostasis, the immune system is able to induce tolerance to innocuous food antigens while it may also recognize pathogenic bacteria to mount an inflammatory immune response. The education and maturation of the intestinal immune system is the result of millions of years of co-evolution with host-specific microbiota and dietary intake. This results in mutual benefits represented by co-habitation and at the same time it provides protection against pathogens. Disruption of these homeostatic mechanisms can result in undesired immune reactions leading to intestinal disorders, such as inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD). At birth, the transition between the sterile environment of the uterus and the external microenvironment exposes our body to colonization with maternal microbiota and food antigens, which are initially delivered through breast milk. Hence, the newborn's immunity relies upon antibodies and other breast milk components (discussed below) passed on from their mothers. At weaning and upon introduction of solid food in the diet, the dynamic equilibrium that allows the homeostatic co-habitation with non-self antigens is continuously maintained through complex mechanisms, involving the constant shaping and education of the immune system. Food nutrients and bacterial metabolites may have a direct effect on the maturation/differentiation of immune cells. In turn, the immune system exerts active oral tolerance mechanisms to prevent reactions against food antigens and to maintain a tolerogenic environment.

This review provides insights to how the intestinal immune system is educated by oral intake of antigens before and after weaning. We will focus our attention on the impact of breast milk and diet during the development of the intestinal immune system.

The Intestinal Immune System: Overview

The intestine is a complex tissue involving a single layer of intestinal epithelial cells (IECs) separating the external environment from the mammalian host. At the luminal side of the IECs layer, a high concentration of potential antigens, in the form of dietary compounds and commensal microbiota, is found, influencing mammalian physiology. At the opposite side, the intestinal lamina propria contains a diverse array of evolutionary ancient immune cells, including mononuclear phagocytic cells and lymphocytes, the associated enteric nervous system and stromal cells (**Figure 1**). The single layer of epithelial cells that separates the intestinal lumen from the lamina propria contains large numbers of lymphocytes with high antimicrobial and cytotoxic capacity. All these different players interact with each other in order to maintain the proper function of the digestive system, whereas failure in keeping homeostasis has been associated with the etiology of intestinal disorders, such as IBD. In the following section, we will introduce the main immune populations present in the intestinal lamina propria and their role in maintaining homeostasis. Then, we will discuss the development of the immune system shaped by breast milk and digested food.

Intestinal Epithelial Cells

The main function of the IEC layer is to provide a physical and biochemical barrier to the external environment. The maintenance of intestinal homeostasis by IECs has been extensively reviewed elsewhere in Ref. (1). In brief, the IECs are composed of Paneth cells, goblet cells, enteroendocrine cells, and enterocytes, which collectively contribute to create the

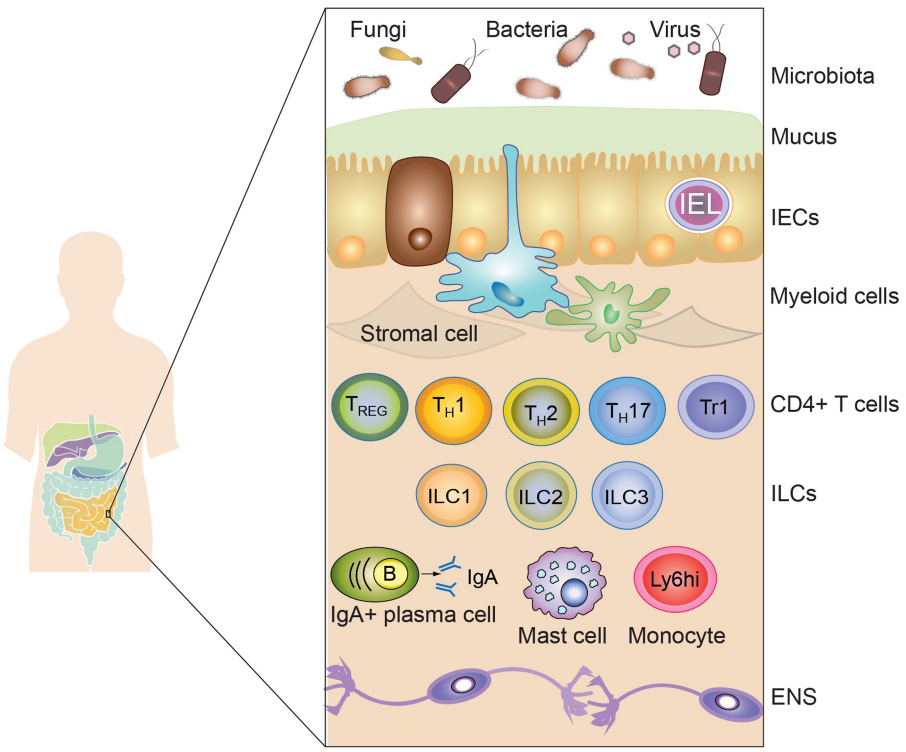

FIGURE 1 | The intestinal immune system. The intestinal immune system is physically separated from the microbiota and dietary compounds by a single layer of intestinal epithelial cells (IECs). Intraepithelial lymphocytes (IEL), residing in the paracellular space between epithelial cells, contribute to the maintenance of the mucosal barrier and to the protection against pathogens. The lamina propria is connective tissue constituted by stromal cells, blood vessels, nerves, and immune cells. Macrophages (Myeloid cells depicted in blue) and dendritic cells (Myeloid cells depicted in green) are strategically located adjacent to the epithelial layer, sampling luminal antigens and orchestrating the innate and adaptive immune response. Other innate immune cells are also present in the lamina propria, including mast cells, monocytes, neutrophils, and eosinophils (not shown). T and B cells (mainly IgA-producing plasma cells) also accumulate in the lamina propria after being primed in the draining lymphoid tissues. Different subsets of $CD4^+$ T cells are found in the lamina propria, such as regulatory T cells (Foxp3-expressing T_{REG} and Tr1) and effector cells (Th1, Th2, and Th17). Finally, innate lymphoid cells (ILC), divided in three main subsets (ILC1, ILC2, and ILC3), are vastly enriched in the gastrointestinal mucosa participating in the protection against pathogens and in the maintenance of intestinal homeostasis. Surrounding the lamina propria and the muscularis mucosa (not shown), the submucosa and the muscularis externa contain nerves belonging to the enteric nervous system (ENS).

first line of defense against microbial invasion. This consists of antimicrobial peptides (AMPs) and the mucus layer. Microfold cells (M cells) are epithelial cells specialized in the sampling and presentation of luminal antigens to the mucosal immune system. M cells are strategically overlaying intestinal lymphoid structures, such as Peyer's patches (PPs) and isolated lymphoid follicles (ILFs). Finally, stromal cells and stem cells, which continuously renew the intestinal epithelium, reside together with Paneth cells within tubular invaginations of the intestinal epithelium, called crypts.

Mononuclear Phagocytes

Mononuclear phagocytes including macrophages (Mφ) and dendritic cells (DCs) are among the most abundant cell types within the intestine. They are strategically located throughout the lamina propria just underlying the single layer of intestinal epithelial cells. Mononuclear phagocytes are sampling luminal content, orchestrating both innate and adaptive immune responses. Under steady state conditions, the lamina propria contains two developmentally different CD11c-expressing primary subsets of mononuclear phagocytes based on the reciprocal expression of the integrin αE (CD103) and CX_3CR1. $CD11c^{hi}CD103^{neg}CX_3CR1^+$ (CX_3CR1^+) cells are considered to be Mφ due to their stationary nature and low stimulatory abilities (2). On the other hand, $CD11c^{hi}CD103^+CX_3CR1^{neg}$ ($CD103^+$) cells are considered to be *bona fide* DCs due to their abilities to migrate to the draining lymph nodes and initiate effective immune responses [reviewed in Ref. (3)]. In addition, these subsets of mononuclear phagocytes have different functions and they cooperate in order to maintain intestinal homeostasis. For instance, CX_3CR1^+ Mφ are specialized in antigen capture from the lumen, however they do not migrate to the mesenteric lymph node (MLN) in steady state conditions (4). By contrast, $CD103^+$ DCs are inefficient in capturing luminal antigens, whereas they efficiently migrate out of the lamina propria to the MLN in a CCR7-dependent manner. Furthermore, $CD103^+$ DCs are able to produce TGF-β and retinoic acid (RA), which equip these cells with the ability to generate inducible regulatory T cells (iT_{REG}) (5, 6). These iT_{REG} are conserved between species (5–7). Induction of gut-homing T_{REG}, likely by RA-producing $CD103^+$ DCs, is a crucial step during the establishment of oral tolerance (discussed below) (8, 9). Together, these cells play a crucial role in distinguishing between innocuous and pathogen-derived antigens and drive both pro- and anti-inflammatory processes. For example, $CD103^+$ DCs selectively express the αv integrin, which is crucial to activate latent TGF-β (10). Activation of latent TGF-β by the αv integrin is physiologically relevant as observed in mouse models lacking αv integrin in the myeloid compartment. These mice develop spontaneous colitis associated with decreased intestinal T_{REG} (11). In addition, CX_3CR1-deficient Mφ show decreased T_{REG} expansion, commonly observed during the establishment of oral tolerance (9). CX_3CR1-deficient mice lack dendrite transepithelial extrusions and have impaired luminal antigen sampling, which result in reduced production of IL-10, typically released upon macrophage sensing of food and/or commensal-derived antigens (9, 12).

Although IL-10 is active in multiple immune cells, including lymphocytes, myeloid cells, and intestinal epithelial cells, it seems that Mφ are the main IL-10 cell target in order to maintain intestinal homeostasis. In fact, mice lacking IL-10Rα, specifically in CX_3CR1^+ Mφ, develops spontaneous colitis (13). This is in agreement with the hyperproduction of inflammatory cytokines and decreased ability to induce CD4 T cells observed by Mφ derived from patients with loss-of-function mutations in IL-10R genes (14). Notably, IL-10 depletion specifically in CX_3CR1^+ Mφ does not result in intestinal inflammation (13), suggesting redundant and/or compensatory sources of IL-10, most likely by type 1 regulatory T cell (Tr1). Hence, these data suggest a model in which Mφ are required to sense IL-10, which might be produced by several different cell types, to become a main tolerogenic cell with a crucial role in intestinal homeostasis. The severity of disease observed in patients with impaired IL-10 signaling underscores the critical role of Mφ and IL-10 at the intestinal barrier. However, the downstream IL-10 signaling pathways involved in imprinting Mφ, with potent tolerogenic properties, are still poorly understood.

Lymphocytes

Naïve B and T cells that accumulate in the intestinal mucosa are primed in gut-associated lymphoid tissues (GALT), such as PPs and mesenteric lymph nodes (MLN). Upon priming within GALT, activated T cells acquire the ability to home to the intestine by expressing the gut-homing chemokine receptor 9 (CCR9) and integrin α4β7. These CCRs bind to the chemokine CCL25 and to the mucosal vascular addressin cell-adhesion molecule (MAdCAM-1), respectively (15, 16), both of them expressed in the small bowel lamina propria. Once lymphocytes, including IgA-producing plasma cells and $CD4^+$ T cells, enter the mucosa they mainly distribute in the lamina propria, with the exception of $CD8^+$ T cells that preferentially migrate to the epithelium (17). $CD4^+$ T cells are divided into subsets, the most abundant found within the intestinal lamina propria are IL-17 producing T helper cells (Th17), Th1 and Regulatory T cells (T_{REG}). T_{REG} include two types of $CD4^+$ T cells; forkhead box P3 (Foxp3)$^+$ T cells and Tr1 cells, which provide the foundation of the tolerogenic immune response. Their relevance during the establishment of intestinal immune homeostasis has been demonstrated by mutations in human *FOXP3,* which are associated with the fatal autoimmune disorder Immunodysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome (18, 19). In mice, disruption of $Foxp3^+$ T_{REG} development and/or function results in intestinal-associated autoimmune and inflammatory disorders (20), likely due to the inability to suppress immune responses against commensal bacteria. Human and mouse Tr1 cells can be identified by the surface markers CD49b and LAG-3 as well as the production of high IL-10 levels (21). Importantly, transfer of Tr1 cells into colitic mice prevents intestinal inflammation, highlighting their immunosuppressive role (21). T_{REG} exert their immunosuppressive functions through several mechanisms, including the production of inhibitory cytokines, such as IL-10 (22), metabolic disruption, expansion of innate lymphoid cells (ILC)1-like NK cells and ILC2s (23,

24), and modulating DC functions (25), all of which have the final outcome of limiting expansion of antigen-specific T cells.

In contrast, Th1 and Th17 cells are mostly associated with pathogenicity during chronic inflammation. Indeed, important efforts to develop drugs targeting the IL-17-Th17 pathway to treat autoimmune diseases have been made (26). In agreement with a pathogenic role, Th17 generation require encounters with antigen presenting cells (APCs) within a pro-inflammatory microenvironment, characterized by the presence of IL-23, IL-6, and IL-1β (27). In addition, intestinal Th17 cells also promote tissue repair and protect mucosal barriers against pathogen colonization, hence contributing to maintain intestinal homeostasis (28). Some studies suggest that their protective and/or pathogenic function depends on plasticity, in which pathogenic Th17 cells might gain immunosuppressive functions (28). In a recent study, using a mouse model of Th17 fate-mapping combined with reporters to visualize the appearance of T_{REG} *in vivo*, it was demonstrated that Th17 cells can transdifferentiate to IL-10-producing T_{REG} to eventually contribute to the resolution of inflammation (29).

Innate Lymphoid Cells

Innate lymphoid cells (ILCs) belong to the lymphoid lineage. In contrast to B and T cells, ILCs lack antigen receptors and do not undergo clonal selection when stimulated. This newly described cell type is vastly present at mucosal surfaces, in particular throughout the GI tract and within ILFs. ILCs have been classified into three subtypes based on their cytokine production and expression of determined transcription factors (30). Group 1 ILCs (ILC1s) are characterized by their expansion in response to IL-12, IL-15 and IL-18, and their production of type 1 cytokines, such as IFNγ and the transcription factor T-bet. Although natural killer (NK) cells also express IFNγ and T-bet, ILC1s are different in that they lack cytolytic activity and possess a separate developmental pathway compared to NK cells. Furthermore, fate mapping of $ID2^+$ or $PLZF^+$ precursor cells distinguished ILC1 from NK cells (31–33). Group 2 ILCs (ILC2s) require the GATA-binding protein 3 (GATA3) and ROR-α transcription factors, expand in response to IL-25, IL-33, and TSLP and produce IL-5, IL-9, and IL-13, typically associated with Th2-responses. Group 3 ILCs (ILC3s) depend on the RA receptor-related orphan receptor-γ (RORγt) transcription factor, respond to IL-1β and IL-23 produced by myeloid cells and secrete high amounts of IL-17A and IL-22, as well as GM-CSF and lymphotoxins (34). Thus, due to their similarities, ILCs are considered the innate counterpart of T helper cells.

Innate lymphoid cells are important mediators of inflammation and exert protection against pathogens, but they are also key drivers of homeostasis under steady state conditions. For instance, ILCs have been associated with worsened *Helicobacter hepaticus*-triggered intestinal inflammation (35). In accordance with this model of pro-inflammatory activities, depletion of ILCs results in significant amelioration of colitis in the *H. hepaticus*-triggered intestinal inflammation (36) and in the *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ ulcerative colitis (TRUC) model (37). Despite the implications with disease, ILC3s have also been implicated in critical protective roles, including defense against the pathogenic bacteria *Citrobacter rodentium* (38, 39), containment of lymphoid-resident commensal bacteria (40) and induction of mucin and antimicrobial peptides (e.g., RegIIIβ and RegIIIγ) (41, 42). Most of these protective functions rely on the production of IL-22 upon stimulation with either IL-23 or IL-1β. ILC3s produce large amounts of this cytokine and administration of exogenous IL-22 in ILC-depleted mice is sufficient to restore homeostasis. In addition, ILC2s and ILC3s express major histocompatibility complex class II (MHCII) (43), hence being able to present antigens to $CD4^+$ T cells. In the intestine, the absence of MHC-II on ILC3s results in expansion of commensal-specific $CD4^+$ T cells, which eventually leads to intestinal inflammation (44, 45). MHC-II expressing ILC2s induce the production of IL-2 and IL-4 by $CD4^+$ T cells, thus potentiating type 2 immune responses (46). Furthermore, it has recently been proposed that the few resident memory T cells with pathogenic features within the non-inflamed intestine might function as antigen-specific sensors, whereas ILCs might serve to amplify T-cell mediated antigen-specific responses (47). Thus, these newly described cell types play critical roles in maintaining intestinal homeostasis through, among others, shaping the intestinal flora.

At early stages in life, the intestinal immune system is underdeveloped and undergoes immune maturation upon contact with dietary compounds and the microbiota. In agreement with this, germ-free and antibiotic-treated mice show reduced maturation of the intestinal immune system, as seen by decreased numbers of intestinal Th17 cells (48, 49), impaired production of antimicrobial peptides, and reduced IgA secretion, all deficiencies that are rescued upon bacterial colonization (50). Besides regulating the diversity of the microbiota, dietary compounds may directly influence the development of the immune system. In the following section, we discuss how the external environment, including dietary compounds and the commensal microbiota, shapes the intestinal immune system.

Breast Milk Shaping Mucosal Immunity

Breast milk is not only a primary source of nutrition, it also helps the child develop a proper immune system. Breast milk is a complex body fluid composed of a large diversity of molecules, cells, and extracellular vesicles. Already in 1892, Paul Ehrlich showed that immunity against plant toxins was transferred to the fetus *in utero* and via breast milk. This turned out to be antibodies protecting the child, leading to the term "passive immunity," which gave him the Nobel Prize and paved the way for modern immunology. In addition to protection to pathogens, breast milk also contributes to establish intestinal tolerance, likely through the action of breast milk-derived components (**Figure 2**), such as immune cells, cytokines, anti-bacterial proteins, probiotics, and extracellular vesicles (51, 52). The role of breast milk in shaping intestinal immune maturation and contributing to immunological tolerance will be discussed below.

Duration of Breast-Feeding

There is great controversy, especially in industrialized countries, regarding the duration of breast-feeding and whether

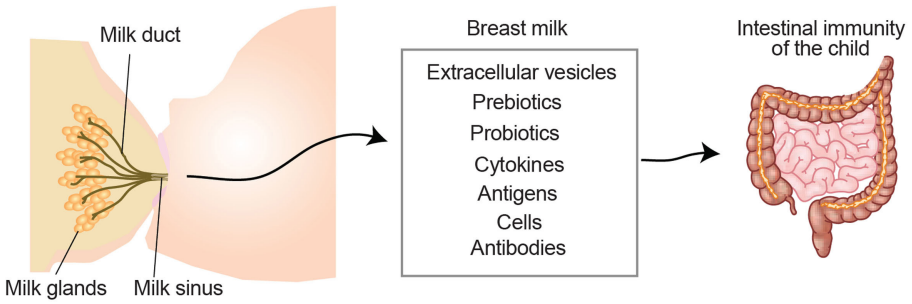

FIGURE 2 | Breast milk-derived compounds with the potential to contribute to the development of the child intestinal immune system. Breast milk contains extracellular vesicles such as milk fat globules and exosomes, which can present antigens in a tolerogenic setting. Pre- and pro-biotics influence the microbiota contributing to symbiosis and intestinal protection. Milk-derived cytokines, which may differ depending on the health status of the mother, have the potential to directly promote the immune system development of the child. Milk-derived antigens have been shown to both induce tolerance and allergies in the lactating child. White blood cells from the mother can also be transferred through the milk and exert functions in the child (e.g., production of cytokines or antibodies). Milk-derived antibodies are originated from the mother and support the child with a passive immunity during the first months of life.

partial or exclusive breast-feeding would be more beneficial. The World Health Organization (WHO) and UNICEF recommend exclusive breast-feeding up to 6 months of age [WHO, Guiding Principles for Feeding Non-Breast-Fed Children Aged 6–24 Months of Age WHO, Geneva (2005)], and countries of the European Union and EFTA recommend exclusive breast-feeding for at least the first 4–6 months (53). In wealthy countries, where early infections, including life-threatening diarrhea are scarce, exclusive versus partial breast-feeding is likely not as important as in developing countries. In contrast, some believe that food allergies can be reduced by introducing solid food under the protection of breast milk.

Several studies have addressed the question regarding the correlation between breast-feeding and allergy development. Most of these studies suggest that breast-feeding may protect against allergies, at least when comparing with formula-feeding (54, 55). However, differences in genetics, exposure to allergens, pathogens, commensals, and smoking, as well as lifestyle, may lead to different conclusions. For example, in a Danish study exclusive breast-feeding duration was associated with increased risk of eczema development in children under the age of 2. In contrast, the risk for developing wheezy disorder in these children was reduced, even with maternal heredity for asthma (56). The exact immunological mechanisms are not easily extracted from epidemiological correlations. In addition, in a review revising the literature regarding breast-feeding and allergies between 1966 and 2001, only 56 out of 4323 studies were considered conclusive (57).

Studies on the relation between autoimmune diseases and breast-feeding are fewer but seem more conclusive for a protective effect of breast-feeding. Retrospective [reviewed in Ref. (58)] and prospective studies have shown that longer breast-feeding is protective for type 1 diabetes (59). Also IBD [meta analysis in Ref. (60)] and multiple sclerosis (61) prevalence are lower in individuals with longer breast-feeding history, indicative of an anti-inflammatory role of breast milk.

Immune-Regulating Components in Breast Milk

During pregnancy, the mammary gland undergoes large modifications in order to transform into a milk-secreting organ. A branching network of ducts that are made up by a single epithelial cell layer and ending up in lobulo-alveolar cavities make up the lactating mammary gland. Surrounding the alveoli are myoepithelial cells, which when stimulated by oxytocin release, contract and enable milk ejection into the cavities (62). The first milk, from delivery to 5 days post-partum, is called colostrum and is produced in reduced amounts and the composition is lower in fat and lactose compared to mature milk (63–65). This is compensated by the colostrum being rich in immunologic components such as lactoferrin, secretory IgA (sIgA), developmental factors (e.g., EGF, TGFβ1, TGFβ2), and other cytokines (65, 66). Hence, the primary function of colostrum might not be nutritional but rather immunologic and trophic (64, 65). Some of the components with potential immunoregulatory functions are discussed below.

Antibodies

The majority of antibodies in milk are IgA, but breast milk also contains IgG and IgE. IgA serves as a first line of defense of mucosal sites to provide a passive immunity to the child who has no IgA production of its own during the first months after birth (67–70). Interestingly, specific antirotaviral IgA has been found in colostrum and mature breast milk and furthermore in infant stool. These antirotaviral antibodies are likely to provide a passive protection in the child (71). Therefore, the transferred sIgA is an important barrier protecting the newborn from intestinal and respiratory pathogens (72). Other studies have shown that maternal sIgA affects the development on gut microbiota in the offspring (73), which in turn affects the immune development. In relation to allergy, it was shown that high IgA levels in the feces of the infant were associated with lower risk for allergy development (74). In addition, allergen-specific immunoglobulins have been detected in neonates after allergen exposure in mice (75) and

humans (76). Whether the IgA work as a decoy receptor for the allergen, or if it is the effect on the microbiota, is still unresolved.

Mouse models support the role for breast milk immunoglobulins and/or B cells in the development of the immune system and allergy development. In a B cell deficient mouse model, it was shown that maternal B cell immunity is important for protecting the offspring against allergic airway disease (77). The transfer of immunity was shown to be accompanied by transfer of antigen-specific IgA and IgG. Another study demonstrated that recombination activating gene-2 (Rag-2) deficient mouse pups (lacking B and T cells) had different immune responses if they received milk from wild type or Rag-2$^{-/-}$ mothers (78), indicating that the lymphocytes from the mother play a crucial role transferring immunity to the progeny. Whether the effects were due to immunoglobulins, cells or both could not be distinguished in this study and remains to be investigated (78).

Cells

Cell exchange between the mother and the fetus occurs already *in utero*, and this results in microchimerism in the child (79). This also continues after birth through breast milk (80). The most abundant cell type in human breast milk is leukocytes, including granulocytes, monocytes, lymphocytes, and Mφ (81, 82). In colostrum, Mφ are the major cells (40–50%) followed by neutrophils (40–50%) and lymphocytes (5–10%) (83–85). Interestingly, breast milk can also contain pluripotent stem cells (86), although their fate within the newborn needs to be investigated. Several studies, in mice, rats and primates have shown that milk-derived leukocytes might be taken up by the offspring where they can survive and exert their functions in the newborn stomachs. The less acidic newborn stomachs might provide a more permissive microenvironment for leukocyte survival compared to adult stomachs (87–89).

Breast milk-derived Mφ are more comparable to tissue resident Mφ, as they have higher HLA-DR expression and are able to carry MHC class II antigens to a higher extent than the monocytes found in blood (90). In addition, they express activation markers such as Leu-M3 and Leu-M5 (90). Neutrophils also seem to be more activated in milk compared to those in blood (91). Moreover, breast milk-derived Mφ also carry intracellular IgA, which can be released upon stimulation (92). Breast milk-derived T cells show a more terminally differentiated and memory phenotype compared to circulating T cells (93). Most breast milk-derived T cells express mucosal homing markers, such as the integrin αE (CD103), integrin β7, CD49d and CCR9 suggesting that they were primed in GALTs and then migrated to the mammary gland (94, 95). Furthermore, γδ$^+$ T cells, which are commonly found in the intestinal mucosa, are also enriched in breast milk compared to blood (96). Breast milk-derived B cells are also different to those circulating in blood. For instance, breast milk-derived B cells show a more activated phenotype compared to those found in peripheral blood (97, 98). Interestingly, IgA-secreting B cells localized in the intestine can home to the mammary glands (99). This phenomenon seems to be dependent on CCR10 (or CCR3), since up-regulation of CCL28 in the mammary gland leads to the recruitment of antibody-secreting cells and to an increased production of IgA in the milk that is blocked using anti-CCL28 antibodies (100). This mechanism might provide breast milk with intestinal microbiota-specific IgA, but other soluble factors from the B cells such as cytokines could also play a role. Thus, these observations suggest that lymphocytes might be educated at intestinal sites to recognize commensal bacteria, traffick to the mammary gland and transfer commensal bacteria-specific immune responses through breast milk to the newborns.

Antigens (Food Allergens/Respiratory Allergens)

Food allergens have been extensively detected in human breast milk. A randomized double-blind cross-over study showed that ovalbumin (OVA) can be detected in human breast milk in a dose-dependent way, up to 8 h after egg intake (101). Other food allergens can also be transferred via breast milk, such as cow milk proteins (102) and the peanut allergen Ara h 6, which can be rapidly transferred to breast milk just 10–20 min after ingestion of peanuts (103). This suggests that food allergens are not fully degraded in the digestive tract of the mother, and further that active allergens can be transferred via breast milk. A comparison between suckling and formula-fed rat pups showed that early introduction of food antigens together with milk shifted the cytokine milieu in a tolerance inducing way (104), which suggests an important early mechanism for development of oral tolerance. Breast-fed pups had higher IFN-γ levels and elevated CCR7$^+$ and Foxp3$^+$ cell numbers compared to formula-fed rats (104). This in turn led to an increased secretion of IL-10 by T_{REG} proposing that these mice have a greater potential to induce a tolerant local gut environment. In agreement, Yamamoto et al. showed in a mouse study that providing lactating dams with food antigens led to induction of oral tolerance to food antigens in the pups (105). Particularly pronounced protection was observed in the offspring of sensitized dams, who had higher antigen specific IgG1 levels in the breast milk. Subsequently, the offspring of the sensitized mothers showed higher amount of antigen specific IgG1 levels in their plasma. Transferred antigens have also been shown to induce tolerance in the offspring. This was observed in mouse pups that were given human breast milk containing peanut allergen, which resulted in partial oral tolerance to the antigen (103). Not only food antigens have been found in human breast milk, but also respiratory antigens such as Der p 1 allergen from house dust mite (106). However, in contrast to food antigens, Der p 1 in milk was shown to promote sensitization when the human milk was given in a mouse model of asthma (106). This discrepancy could be due to either the xenobiotic nature of the experiment or to the nature of this antigen, as it has enzymatic activity and can damage the epithelial barrier (107). In an OVA model, Verhasselt et al. showed elegantly that OVA areosols could be transferred via milk and induce tolerance in the pups, and that this was dependent on TGF-β (108). However, it is difficult to rule out if OVA have been digested to some degree. In addition, the presence of the antigen in the gut might contribute to induce tolerance.

Cytokines and Other Soluble Factors

Several chemokines and cytokines, which might have a major impact on the mucosal and lymphoid tissues in the child have

been found in human breast milk (109). IL-6 and TGF-β have been identified as the most abundant cytokines present in breast milk (110). For instance, breast milk-derived IL-6 has been shown to be crucial for IgA production from milk mononuclear cells (111). With potential implications in allergy, IL-4 can be detected in both colostrum and mature breast milk, with elevated levels in colostrum from allergic mothers compared to non-allergic mothers. Similarly, IL-8 is found in higher amounts in allergic mothers compared to non-allergic mothers (112). In contrast, the immunosuppressive cytokine IL-10 has also been detected in breast milk (113). *In vitro* studies have shown that an anti-proliferative effect of milk on peripheral blood mononuclear cells (PBMC) was reduced when adding anti-IL-10 antibodies, demonstrating IL-10 activity in the milk (113). Breast milk-derived TGF-β might also play an important role inducing intestinal tolerance. In agreement, breast milk-derived TGF-β acts on T cells, which eventually confer protection to develop allergies (108). Finally, IL-1 receptor (IL-1R) antagonist has been found in human breast milk. The fact that mice with colitis are protected similarly if they receive human milk or formula supplemented with IL-1R antagonist (114), suggest that IL-1R signaling can have a major role in tolerance induction in the offspring.

The soluble protein CD14 can work as a receptor for lipopolysaccharide and can be transferred through breast milk from rat dams to the pups (115). CD14 is up-regulated after bacterial exposure and is secreted by innate immune cells such as monocytes as well as epithelial cells (116). The association between low exposure to CD14 via the breast milk and increased risk for atopy has been observed in some cohorts (117) but not in others (118), and it remains to be further studied whether breast milk-derived CD14 modulates immunity in the offspring. In addition, the presence of complement factors (119), free radicals and antioxidants, such as β-carotene (120) and α-tocopherol (121) in milk are also likely to modulate the microbiota and intestinal immunity of the child.

Factors Affecting the Microbiota

It has been suggested that bacteria from the maternal gut can be transported by mononuclear cells to the mammary gland and transferred to the breast-fed infant (122, 123). Human breast milk contains a large diversity of probiotic (having a positive health effect) bacteria, such as, *Lactobacillus rhamnosus, Lactobacillus gasseri, Lactococcus lactis, Leuconostoc mesenteroides*, *and Bifidobacteria*. Therefore, breast milk is an important source of bacteria for the undeveloped gut of the infant (124–127). Breast milk-derived probiotics possess the ability to positively modify the gut microbiota of the infant (e.g., to help improving the health of the infant gut). Furthermore, in a large population-based pregnancy cohort, an association has been shown between reduced risk of atopic eczema in infants and the consumption of probiotic milk of mothers during pregnancy (128). Therefore, probiotics might reduce the risk of atopic eczema (128).

Oligosaccharides are prebiotics (carbohydrates serving as nutrient for probiotics) that are abundant in milk, which also alter the gut flora (129). High levels of oligosaccharides in breast milk correlate with higher diversity of *Bifidobacterium* species, and inhibit the adhesion of pathogenic bacteria (130), hence showing protection to infection. Moreover, Lactoferrin is another component of milk with bactericidal properties against many pathogenic bacteria (131). However, Lactoferrin can also promote the growth of *Bifidobacteria* species (132), leading to an altered gut flora. The medium-chain saturated and long-chain unsaturated fatty acids present in breast milk have also been shown to be anti-microbial (133) (see above). In addition, lysozyme will also affect the gut flora of the infant since it has both anti-bacterial and anti-viral activities (134, 135). This is probably contributing to the anti-pathogenic effect of breast milk, but the effect on the commensal flora is still unclear.

Extracellular Vesicles

The mammary epithelial cells secrete milk through five different secretory pathways (62), whereof the milk fat globule pathway is specific for mammary epithelium (136). This pathway is a budding process, which generates lipid-containing vesicles, milk fat globules (MFG) surrounded by a lipid membrane, and is a way to release the fat without the risk of clumping. The MFGs has a protective effect against microorganisms (137–139), which comes partly from the triacylglycerol rich-core, but also from the membrane, which contain pH resistant glycoproteins such as mucin-1 (MUC-1), MUC-X, and lactadherin (138, 139). MUC-1 has been shown to attenuate epithelial inflammation (140), in agreement with the role of mucins creating a protective shield in the newborn gut against bacteria and viruses (141). Another type of extracellular vesicle that can be found in breast milk is exosomes (142), which are nano-sized membrane vesicles, which originate from the endosomal pathway. Exosomes contain a large diversity of lipids, proteins, and several kinds of RNA (143, 144). Importantly, the presence of mRNA (145) and miRNA (146) has been detected within breast milk-derived exosomes. Some of the proteins are common for all exosomes, regardless of their cellular origin, such as proteins involved in multivesicular body biogenesis or membrane transport and fusion, and tetraspanins such as CD9, CD63, and CD81 (147). Exosomes also contain cell-specific proteins, such as MHC class I and II as well as co-stimulatory molecules on their surface, when derived from APCs (148).

Virtually all cells release exosomes, which have both immunostimulatory (149) and immunosuppressive functions (150). First reported to have an immunostimulatory function, were exosomes released by B cells, which express MHC class II and are therefore equipped with the ability to induce antigen-specific $CD4^+$ T cell responses *in vitro* (149). Importantly, using DC-derived exosomes harboring MHC class I, induction of $CD8^+$ T cell responses was shown *in vivo* (151). The ability of exosomes to indirectly or directly stimulate T cells has been documented by several other studies (152–154).

Our studies have shown that breast milk-derived exosomes are likely to have an immunosuppressive role, as observed in PBMC cultures *in vitro*, in which the presence of breast milk-derived exosomes were able to inhibit cytokine production and induce $Foxp3^+$ T_{REG} (142). Whether this effect was mediated via DC or the exosomes acting directly on the T cells, is currently under investigation. Cow's milk has also been shown to contain extracellular vesicles carrying TGF-β (155), and bovine milk exosome-derived microRNA was shown to have immunoregulatory effects in *in vitro* macrophage experiments (156), further suggesting an

immunosuppressive role for breast milk-derived exosomes. An immunosuppressive function of exosomes derived from the gut epithelium has also been shown in their ability to induce antigen-specific tolerance (150). Exosomes derived from serum of OVA fed rats have been shown to induce antigen-specific tolerance in naïve recipient animals. The origin of these exosomes is believed to be the intestinal epithelium, as exosomes isolated from *in vitro* pulsed intestinal epithelium cells showed the same characteristics as the serum-derived exosomes (150). In addition to intestinal epithelium-derived exosomes, also exosomes of other cellular origins have the ability to induce tolerance. Prado et al. demonstrated that exosomes derived from BAL fluid of antigen-specific tolerized mice could inhibit allergen-induced airway inflammation (157). This was shown by decreased levels of IgE antibodies in mice pre-treated with BAL-derived exosomes from tolerized animals, compared to those treated with control exosomes. In addition, they further showed that these mice, pre-treated with exosomes from tolerized animals, also had a reduced level of IL-10 and a significantly reduced production of IL-5 (157). Whether breast milk-derived exosomes possess similar properties *in vivo* still needs to be investigated.

Although a role for breast milk exosomes in shaping the immune system of the child is plausible, only *in vitro* studies and indirect observations are currently available to support this. In a study comparing allergic and non-allergic mothers, we have shown that allergic sensitization and lifestyle of the mother affects the phenotype of breast milk-derived exosomes. In this study, it was also shown that there is a relationship between the breast milk-derived exosome profile and the sensitization of the child, suggesting that the exosomes play a role in the cell-to-cell communication between mother and child (158). We also observed that MHC class I was low in early milk, and higher in late milk, suggesting that endogenous antigens, e.g., viruses or autoantigens, might be more exposed later after birth. By contrast, since MHC class II was high early and reduced later, allergens transferred from the mother might be more presented early after birth. One could speculate that this differential exposure has evolved to expose the child to different antigens at optimal time points during development. However, new exposure patterns in modern lifestyle might have led to an imbalance and more allergy or autoimmune development. In addition, breast milk-derived exosomes carry MUC-1 and were shown to interact directly with DCs by binding to the DC receptor DC-SIGN, and furthermore blocked HIV infection of DC by blocking the entry of the virus (159). Whether this occurs *in vivo*, with possible delivery of exosome cargo to DCs in the gut or in the circulation, remains to be investigated. However, since exosomes ("tolerosomes") also can be produced by the gut epithelium, and have in this context been shown to be able to induce tolerance, we speculate that also milk exosomes could have this effect (150), delivering tolerogenic signals to the offspring.

Upon weaning, breast-feeding is discontinued whereas dietary intake of solid food increases, exposing our GI tract to a vast range of nutrients and exogenous antigens. Dietary intake and eating habits have a profound impact in shaping the immune system and regulating the microbial composition of the gut. However, a recent study suggested that interruption of breast-feeding together with the mode of delivery (vaginal compared with C-section) are the crucial steps in the definition of microbiota assembly, rather than the introduction of a solid-food diet (160). Nevertheless, a controlled nutritional regimen is crucial for the proper maturation of a functional immune system and to allow protection against infection and intestinal or systemic disorders (161). Furthermore, paralleling the hygiene hypothesis, a "diet hypothesis" has been proposed to explain the differential incidence of allergic and chronic inflammatory diseases among countries with a similar cleanliness in the environment but with remarkable different diets (162). Education of the intestinal immune system in relation to dietary intake can be outlined into two main processes: (1) The sensing of specific food metabolites, which affects maturation, differentiation, and activity of the intestinal immune system. (2) The active mechanisms to induce tolerance toward ingested antigens, a process referred to as "Oral Tolerance".

Dietary Compounds Shaping Mucosal Immunity

Our body directly absorbs some dietary metabolites, while others are indigestible by the enzymatic repertoire encoded by our genome. Therefore, microbial digestion of these metabolites is needed for their absorption (163). This is likely reflected by a spatial distribution of these food metabolites along the GI tract. For instance, Vitamin A or food ligands for the AhR receptor (see below) are enriched in the proximal small intestine (e.g., duodenum). By contrast, short-chain fatty acids (SCFA), which are metabolites of indigestible fibers, require bacterial enzymes to be digested and absorbed, hence being more concentrated in the large intestine (16). Consistently the effect of these food components on the education of immune cells follows the same pattern of distribution. (16) (**Figure 3**). As an example, SCFA promote the generation of $CD4^+$ T_{REG} cells that are enriched in the colonic tissue, whereas AhR ligands drive the differentiation of Th17 cells that in turn are more prevalent in the small intestine. These examples highlight the concept that the small intestine and colon are two different compartments composed of different metabolites, organisms and cell types. In the following sections, we will discuss how food metabolites educate our intestinal immune system. We will discuss the role of food components that are directly absorbed by our body ("Unmodified dietary components") and dietary antigens that require bacterial metabolic processing ("Commensal-derived food metabolites").

Unmodified Dietary Compounds

Some food components do not require metabolic processing by commensal bacteria in order to be absorbed and digested by our body.

Vitamin A

Deficiency in Vitamin A and its metabolite retinoic acid (RA) in the diet has been associated with increased HIV pathogenesis, poor responses, and increased susceptibility to infections, both at the GI tract and in the lungs (164, 165). The dietary sources of Vitamin A encompass animal products (such as human milk, liver, and egg yolk) containing Vitamin A in the form of retinyl

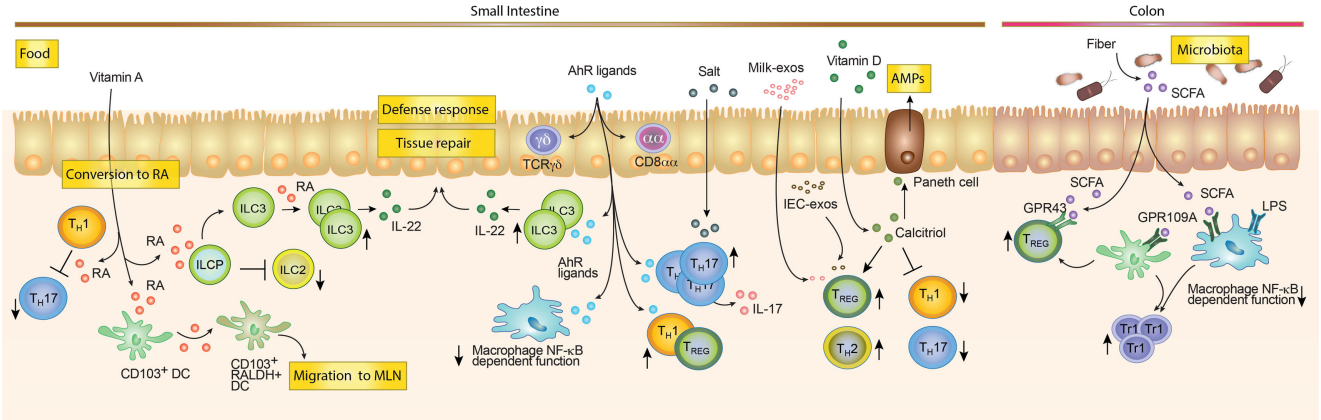

FIGURE 3 | Dietary compounds contributing to the development of the intestinal immune system. Food components acting directly on immune cells such as Vitamin A and D, salt and AhR ligands are enriched and mostly absorbed in the small intestine. Vitamin A can be converted by RALDH expressing cells into Retinoic Acid (RA). RA can modulate the function of different cells. For example, it can inhibit Th1 cells to become pathogenic Th17 cells, block ILC2s development while promoting ILC3s differentiation and expansion and imprint DC with the ability to produce RA. While in the mesenteric lymph node (MLN), RA-producing DCs may induce gut tropism on T and B cells, as well as promoting T_{REG} differentiation (not shown). AhR ligands favor the maintenance of intraepithelial lymphocytes (e.g., TCRγδ and CD8αα T cells). Furthermore, they promote Tr1 and T_{REG} differentiation, inhibit NF-κB signaling in macrophages and promote the expansion of ILC3s. Salt rich diets and AhR ligands are associated with expansion of Th17 cells. Milk-derived exosomes (Milk-exo) and epithelial cell-derived exosomes (IEC-exo) have been shown to promote T_{REG} differentiation/expansion. Although, the precise mechanism and if this occurs in the intestine *in vivo* is still unknown. Vitamin D is converted into the active metabolite Calcitriol ($1,25(OH)_2D_3$), which may promote T_{REG} and Th2 cells while blocking Th1 and Th17 differentiation. In addition, Calcitriol enhances the production of anti-microbial peptides by Paneth cells. Dietary compounds, which require bacterial enzymatic digestion, may preferentially exert immunoregulatory functions in the colon. Dietary fibers are metabolized by the microbiota, which leads to the production of short-chain fatty acids (SCFA) metabolites. SCFA promote T_{REG} responses through GPR43 and indirectly by triggering GPR109A on colonic myeloid cells (Mφ and DCs). In addition, the anti-inflammatory effect of SCFA is mediated by the inhibition of histone deacetylases (not shown), NF-κB signaling and the inhibition of the LPS-induced inflammatory cascade.

esters and food containing Vitamin A precursors, or carotenoids (such as yellow and green leafy vegetables). Upon absorption in the proximal small intestine, retinyl esters undergo a complex metabolic pathway that might lead to the oxidation of the Vitamin A metabolite, retinal, into RA. RA functions are then mediated by the binding to the RA nuclear receptors (RARα, RARβ, and RARγ) heterodimerized with retinoid X receptors (RXRα, RXRβ, and RXRγ) (166). The crucial role of RA orchestrating mucosal immunology began to be elucidated by the seminal article published a decade ago by Iwata and colleagues, showing that RA was necessary and sufficient to induce leukocyte trafficking to the gut mucosa (167). Subsequent work has shown that RA induces gut tropism in B cells and promotes the induction of IgA-producing plasma cells at mucosal surfaces (168). Furthermore, RA acts as an adjuvant to induce $Foxp3^+$ T_{REG} and Th17 differentiation [reviewed in Ref. (169)]. In addition, recently Noelle and colleagues showed that RA action on RARα represses the reprograming of Th1 to pathogenic Th17, hence playing a role in Th1–Th17 plasticity (170). The effect of RA might depend on the microenvironment. In a pro-inflammatory milieu characterized by the presence of IL-15, RA promotes inflammatory cellular and humoral responses to fed antigens (171).

Retinoic acid might also indirectly act on the immune system. This was observed in mice reared on a Vitamin A deficient diet (VAD), which results in disruption of number and composition of the gut microbiome (172). In particular, decreased segmented filamentous bacteria (SFB) were accompanied by a reduced proportion of Th1, Th17, and Th2 cells (173).

Recently, a critical role of RA in ILC homeostasis has been demonstrated. For instance, mice reared on a VAD are characterized by increased numbers of ILC2s and reduced numbers of ILC3s as well as ILC3-derived cytokines, such as IL-17 and IL-22 (173). Interestingly, the transfer of RARα-deficient ILCs progenitors recapitulated the same phenotype, suggesting that RA intrinsically suppresses ILC2 development. Besides its role in differentiation, RA modulates IL-22 production possibly by direct engagement of the RARγ with the IL-22 promoter (174). Another recent report demonstrated that RA regulates the differentiation of lymphoid tissue inducer (LTi) cells, which are essential for the development of secondary lymphoid organs (175). As a consequence, the availability of RA *in utero* has an impact on the education of the newborn immune system and protects against infection in adulthood (175). Thus, RA plays a crucial role in ILC homeostasis, which is then reflected by increased susceptibility to bacterial infections, intestinal inflammation, and the overall fitness of the intestinal immune response (173, 174).

In adults, the sources of RA have been extensively studied and reviewed elsewhere in Ref. (176). The limiting enzymes in RA metabolism are retinal dehydrogenases (RALDHs), which are highly expressed by intestine-resident $CD103^+$ DCs, epithelial cells and stromal cells, as well as by $CD103^-$ $CD11b^+$ DCs in the skin and lung (166). Expression of RALDH enzymes is crucial to metabolize RA, since cells lacking these enzymes are not able to produce RA (177). Interestingly, gut-homing expressing DC precursors migrate from the bone marrow to the small bowel, where they acquire tolerogenic properties (178, 179). Once DC

precursors reach the intestine, they might differentiate and sense RA to induce the expression of RALDH enzymes, with the subsequent *de novo* production of RA (179). The role of RA in educating intestinal DC is in agreement with the levels of Vitamin A in a proximal to distal gradient, which correlates with the abilities of DC to produce RA depending on their location in the intestinal tract (177). For instance, DCs obtained from the proximal small bowel express higher *raldh2* mRNA levels compared to distal small bowel-derived DCs, correlating with their abilities to induce gut-homing receptors on T cells, conversion of Foxp3$^+$ T_{REG}, and induction of IgA class switch on B cells (177). These examples highlight the concept that the availability of nutrients locally shapes the immune system. Recent efforts are pointing toward the requirements of vitamin A for proper development of the immune system with the consequent relevance for protection against infection and inflammatory disorders. Future studies exploiting conditional knockout of RA receptors in different immune cell subsets will be crucial to better understand the pleiotropic effects of Vitamin A on the immune system.

Vitamin D

In addition to Vitamin A, Vitamin D and its active metabolite calcitriol ($1,25(OH)_2D_3$), can influence the immune system beyond its metabolic role in calcium and phosphate homeostasis. The major source of Vitamin D is the photochemical conversion of 7-dehydrocholesterol to cholecalciferol (Vitamin D3) upon skin exposure to UV light. However, some food such as oily fish are enriched in Vitamin D and serve as a relevant source. Calcitriol, the active form of Vitamin D, results from a complex metabolic pathway involving hepatic and renal enzymatic activity (180). The biologic function of Vitamin D is mediated via binding to the hormone nuclear receptor Vitamin D receptor (VDR) heterodimerized with the RXR, which in turn promotes the transcription of Vitamin D responsive genes by binding their promoter region. Besides its role in regulating intestinal, renal and skeletal absorption of calcium and phosphate and in regulating blood pressure, VDR is also expressed by immune cells that in turn are equipped with the enzymatic machinery to produce calcitriol locally (181). The general positive effect of Vitamin D on the adaptive immune system is anti-inflammatory, as observed in studies showing inhibition of B (182) and T cells proliferation (183) and promotion of a shift from the Th1/Th17 lineage toward a T_{REG}/Th2 phenotype (184–186). Consistently, calcitriol action on innate immune cells results in the blockade of production of pro-inflammatory cytokines in CD40L-activated monocytes (187) and the promotion of an immature/regulatory phenotype on DCs (188). The immunoregulatory function of Vitamin D, together with the observation of a higher incidence and prevalence of IBD in northern European countries with lower sunlight exposure, prompted the implication of hypovitaminosis D in the pathogenesis of this disease (189). Indeed, low Vitamin D levels were observed in pediatric Crohn's disease patients (190) and VDR gene polymorphisms were associated with increased susceptibility to IBD (191). In addition, Vitamin D plays a role in modulating cell homing marker expression. DC-dependent production of calcitriol results in the induction of skin-homing receptors (CCR10) and in the inhibition of gut-homing molecules (α4β7 and CCR9) on activated T cells (192). However, whether this redirection of immune cells is implicated in IBD development remains to be addressed. In the murine setting, IL-10$^{-/-}$ mice (an established mouse model for IBD) crossed with VDR$^{-/-}$ mice displayed exacerbation of colitis and fulminating inflammation when compared with VDR-sufficient control mice (193). Similarly, VDR$^{-/-}$ mice were shown to be more susceptible to Dextran Sodium Sulfate (DSS)-induced colitis due to impaired healing and decreased epithelial junctional complexes, suggesting a role for Vitamin D in the maintenance of the intestinal mucosal barrier (194). Interestingly, VDR$^{-/-}$ mice are characterized by increased bacterial load in the intestinal mucosa. This is most likely due to a VDR-dependent transcriptional regulation of the autophagy gene ATG16L1 in Paneth cells, which are important for the production of antimicrobial peptides (195). Although preclinical and clinical observations point toward an involvement of Vitamin D in the pathogenesis of IBD, further studies addressing whether hypovitaminosis D is a cause or a consequence are needed. IBD patients are characterized by inflammation-dependent intestinal malabsorption of diet-derived Vitamin D (180). However, recent prospective studies suggest that newly diagnosed IBD patients show Vitamin D deficiency whereas higher Vitamin D status correlates with a lower risk of developing Crohn's disease (196).

AhR Ligands

Another relevant dietary constituent enriched in the small intestine is represented by aryl hydrocarbon receptor (AhR) ligands. AhR is a widely expressed basic helix-loop-helix transcription factor, belonging to the PAS domain-containing superfamily. Interestingly, its expression on immune cells plays a pivotal role in the development and differentiation of the immune system (197). Ligands of AhR encompass xenobiotics such as environmental pollutants, endogenous ligands such as tryptophan metabolites (e.g., FICZ) and dietary ligands contained in cruciferous vegetables like broccoli and cabbage (198). Remarkably, a recent report showed that AhR might also act as a sensor of PAMPs by binding bacterial pigmented virulence factors (viz. phenazines from *Pseudomonas Aeruginosa* and naphtoquinone phthiocol from *Mycobacterium Tubercolosis*) and enhancing host immune response against pathogens (199). AhR ligands have been linked to the maintenance, but not the embryogenesis, of intraepithelial lymphocytes (e.g., TCRγδ and CD8αα T cells) (200) as well as differentiation and effector function of Foxp3$^+$ T_{REG}, Tr1, and Th17 cells (201–203). Recent findings highlighted a crucial role for AhR signaling in the innate lymphoid cells compartment. AhR deficiency dampens the postnatal, but not the fetal, development of RORγt$^+$Nkp46$^+$ ILC3, thereby affecting IL-22 mediated protection against *C. rodentium* infection (204). Interestingly, AhR-dependent IL-22 expression by ILC3 was shown to be crucial to avoid the expansion of SFB and the induction of Th17 cells in the intestine, suggesting a cross-regulatory role of AhR that directly promotes Th17 cell differentiation and indirectly control their expansion by regulating the activity of ILCs (205). The requirement of AhR-mediated signaling during postnatal development was also outlined by

two studies demonstrating an AhR-dependent development of CCR6$^-$CD4$^-$T-bet$^{+/-}$ RORγt$^+$ ILCs to generate cryptopatches and isolated lymphoid follicles (206, 207). Furthermore, AhR expression on peritoneal Mφ has been associated with hyporesponsiveness to LPS achieved by NF-κB inhibition (208). Hence, AhR ligands may target Mφ to maintain tolerance against the non-self repertoire enriched in the GI tract. In addition, AhR negatively regulates NLRP3 inflammasome activity (209), further suggesting a role in dampening intestinal inflammation. To better determine the role of AhR on specific intestinal immune cells, additional studies exploiting an AhR ligand deficient diet, conditional ablation of AhR and a discrimination of microbial derived AhR ligands are needed.

Salt (NaCl)

It has recently been shown that salt, which is contained in high concentration in the western diet, induces pathogenic Th17 cells in the intestinal lamina propria (210), and hence providing an explanation for the link between the western life style and the high incidence of IBD observed in developed countries (189). Mechanistically, the serine-threonine kinase SGK1 (serum glucocorticoid kinase-1), which is important in controlling Na^+ transport and salt homeostasis, is induced in T cells activated in Th17 polarizing conditions. SGK1 signaling blocks Foxo1, which is a repressor of IL-23R. Thus, SGK1-deficient cells display normal primary differentiation of Th17 cells, but impaired IL-17 production upon IL-23 restimulation (210). Consistently, WT mice fed with a high salt diet were characterized by an increased frequency of Th17 in the SI-LP when compared to mice lacking SGK1 in the CD4$^+$ compartment (210).

Commensal-Derived Food Metabolites

Some dietary components require special enzymes for their digestion; those that are lacking in our genome are instead provided by our microbiota (211). Mice reared under germ-free conditions display an altered metabolism with accumulation of undigested fibers and of primary bile acid products (212). The influence of commensal bacteria in regulating the availability of diet-derived nutrients and metabolites and their impact on the immune response have recently been reviewed elsewhere in Ref. (213), therefore we will in brief only discuss recently published results.

Short-chain fatty acids

Indigestible dietary fibers, which are present in fruits, vegetables, and cereals, represent the prototype of food metabolites that require bacterial enzymatic digestion in order to be biologically available. Fibers are processed by bacterial fermentation into SCFA products (mainly acetate, propionate and butyrate), which are enriched in the proximal colon in agreement with the higher bacterial load (16). Although butyrate has been associated with a pro-tumorigenic role in the etiology of colorectal cancer (214), it has also been shown that administration of butyrate to mice, which are genetically susceptible to small intestinal cancer and kept under a high fat diet, is able to attenuate tumor progression (215). Overall, the effect of a fiber-enriched diet results in the induction of an anti-inflammatory environment and thus provides a protection against inflammatory and allergic disorders. In line with this, *in vitro* butyrate-treated DCs produced lower levels of inflammatory cytokines, such as IL-12 and IFNγ (216). Furthermore, butyrate modulates intestinal macrophage function through histone deacetylase (HDAC) inhibition and downregulation of the LPS-induced inflammatory cascade (217). Moreover, butyrate binding to GPR109A, which is a receptor for niacin in colonic Mφ and DCs, results in the generation of Foxp3$^+$ T_{REG} and IL-10 producing Tr1 cells (218). *In vivo*, the most prominent effect of SCFAs is the expansion of Foxp3$^+$ T_{REG} cells in the colonic lamina propria. SCFA-mediated effects on the immune system are mostly exerted through inhibition of HDAC and signaling downstream GPR43 and GPR41 receptors (213). A SCFA-enriched diet mediates the expansion of Foxp3$^+$ T_{REG} in the colon in a cell intrinsic and GPR43-dependent manner (219), whereas previous results showed the same expansion mediated by inhibition of deacetylation of the Foxp3 promoter (220). Interestingly, from a metabolic point of view, Th17 cells, differently from Foxp3$^+$ T_{REG}, are dependent on the glycolytic pathway (221). A recent study showed that *de novo* fatty acid synthesis is crucial for Th17 cell differentiation, whereas T_{REG} are mostly induced by fatty acid uptake, thus providing a concomitant metabolic explanation of the phenotype observed *in vivo* upon changes in the dietary intake (222). Consistently with the enlargement of the Foxp3$^+$ T_{REG} pool in the colon, mice fed with a fiber enriched diet showed decreased T cell transfer-mediated colitis, which depends on a direct effect of SCFA on Foxp3$^+$ T_{REG} rather than *de novo* Foxp3$^+$ T_{REG} generation (219). Using a chemically induced murine model for colitis, Berndt and colleagues demonstrated that the route of administration is crucial to determine a protective or a harmful role of butyrate (223). The analysis of feces from human patients with IBD compared with healthy subjects revealed decreased levels of acetic, butyric, and propionic acids, thus suggesting a protective role of SCFA in the pathogenesis of IBD (224). Moreover, the fecal microbiota of patients with IBD showed decreased levels of *F. Prausnitzii*, a bacterial species of Firmicutes involved in dietary fiber digestion (225). Nevertheless, the effect of a fiber enriched diet in the treatment of IBD patients has shown opposite results (226–228), possibly depending on the different experimental protocols used in the different studies and on the selection of the patients cohorts (227). A better understanding of the local versus systemic effect mediated by this metabolite is needed to achieve a deeper insight in the mechanisms of action and the possible consequences in the shaping of the immune response in the GI tract and in other compartments of our body. In line with this, a fiber-rich diet is reflected by an increase in the serum level of SCFAs and is associated with a reduced susceptibility to allergic airway inflammation in a GPR41-dependent manner. This protective effect appeared to be mediated by an increased proliferation of CDPs and MDPs in the BM and the subsequent seeding of the lungs with DCs characterized by an increased phagocytic potential but a decreased ability to produce type 2-promoting cytokines (229). Whether this extra-intestinal effect of dietary metabolites is dependent on a direct sensing of SCFAs by BM precursors or whether it is an indirect effect mediated by education of immune cells in the intestine, followed by delivery of signals to the BM, still needs to be investigated.

Oxysterols and Bile Acids

Food enriched in oils and fat is an important source of dietary lipids, mainly triglycerides and cholesterol, which exert important roles in regulating the immune system (230). Metabolism of cholesterol gives rise to oxysterols and bile acids, key players in the regulation of nutrient absorption and lipid digestion through the activation of the nuclear receptors LXR (LXRα and LXRβ) and FXR, respectively. Commensal bacteria are important regulators of the synthesis of bile acids by converting primary into secondary bile acids and by favoring their excretion. Bile acids binding of FXR-RXR heterodimerized receptor expressed on lamina propria CD11b$^+$ Mφ results in a decreased induction of pro-inflammatory genes, like IFNγ, IL-6, TNF-α, and IL-1β. Therefore, mice treated with an FXR agonist displayed a milder phenotype of inflammation during DSS-induced colitis (231, 232). Similarly, LXR activation on LPS treated Mφ results in the inhibition of COX-2, NOS, and IL-6 expression with the subsequent reduced inflammation in an *in vivo* model of dermatitis (233). Consistently, LXR-deficient Mφ display an aberrant capacity of apoptotic cell clearance associated with induction of inflammation and breakdown of self-tolerance (234). On the other hand, LXRβ deficiency was associated with an enhanced proliferative capacity of T lymphocytes and lymphoid hyperplasia, implying the importance of LXR and sterol metabolism in lymphocytes homeostasis (235). Furthermore, LXR activation has been associated with impaired differentiation of Th17 cells and protection against experimental autoimmune encephalomyelitis (EAE) in mice (235). Given the need to maintain intestinal homeostasis, metabolism of cholesterol might be crucial to counteract pro-inflammatory exogenous insults highly present in the GI tract. However, the specific target cells sensing oxysterol and/or bile acid to exert homeostatic function is still unresolved. Interestingly, a recent study demonstrated that naturally occurring oxysterols (7β, 27-OHC, and 7α, 27-OHC) are strong RORγt agonists, hence enhancing Th17 differentiation *in vitro* in both human and mice (236). This study was also further substantiated by *in vivo* findings. Mice deficient for CYP27A1, an enzyme responsible for bile acid synthesis and 27-OHC production, showed diminished numbers of Th17 cells among splenic CD4$^+$ and γδ T cells (236), suggesting that oxysterols might, independently of LXR, modulate intestinal Th17 homeostasis.

Overall, the concept of diet as an active player involved in shaping our immune system rather than just being a mere source of energy is broadly accepted. The study of nutritional regimens in different areas of the world paired with the differential incidence of inflammatory intestinal disorders is currently providing important insights in their pathogenesis and etiology. Current efforts point to uncover dietary derivatives associated to cellular and molecular changes leading to intestinal inflammation or reestablishing homeostasis. Moreover, a better understanding of diet-mediated immune effects will be crucial to exploit changes in dietary habits as an important complementary therapeutic approach for the treatment of inflammatory disorders affecting the GI tract.

Oral Tolerance to Food Antigens

As mentioned, the gut mucosa is constantly exposed to commensal microflora and food constituents. Therefore, efficient suppressive mechanisms are needed to avoid undesired inflammatory responses against these innocuous antigens. Oral tolerance is a process characterized by local and systemic hyporesponsiveness to antigens administered via the intestinal mucosa. Defects in proper induction and/or maintenance of oral tolerance might underlie pathological immune responses not only in the gut (e.g., IBD and food allergy), but also beyond the gut (e.g., multiple sclerosis).

Cellular Mechanisms of Oral Tolerance

The induction of oral tolerance relies on a stepwise active mechanism exploited by intestinal immune cells belonging to the adaptive and the innate immune system (237, 238). It is broadly accepted that induction of antigen-specific Foxp3$^+$ T_{REG} in gut draining lymphoid organs, and subsequent migration of these antigen specific Foxp3$^+$ T_{REG} into circulation, are key features to establish oral tolerance. However, in recent studies, a multi-step model for the induction of oral tolerance has been proposed, in which prior to entering into the circulation, Foxp3$^+$ T_{REG} migrate to the gut mucosa with the purpose to acquire immunosuppressive abilities (**Figure 4**) (5). The different steps involved in this process include: (1) antigen sampling by innate immune cells; (2) migration to intestinal secondary lymphoid organs (mostly the mesenteric lymph node) and antigen presentation; (3) generation of Foxp3$^+$ T_{REG} with gut tropism; (4) migration of Foxp3$^+$ T_{REG} to the intestinal lamina propria where they expand and acquire tolerogenic potential; (5) eventual migration of newly generated Foxp3$^+$ T_{REG} into circulation and prevention of inflammation in the periphery (5).

Antigen Sampling

Luminal antigen sampling rely on several mechanisms involving different cell types, including Peyer's Patches-associated M cells, intestinal epithelial cell-mediated intake, and protrusion of dendrites by CX_3CR1^+ Mφ across the epithelial layer (237). However, lamina propria Mφ of the uppermost part of the small intestine are less efficient in antigen presentation and, as mentioned above, do not migrate to draining lymph nodes when compared to CD103$^+$ DCs. Since antigen presentation by CD103$^+$ DCs appeared to be crucial to induce oral tolerance, sampled antigens need to be transferred from CX_3CR1^+ Mφ to CD103$^+$ DCs. Interestingly, Rescigno's group demonstrated that orally delivered antigens, internalized and processed by CX_3CR1^+ Mφ, could be transferred via Connexin 43$^+$ gap junctions to migratory CD103$^+$ DCs in order to allow MHC-II mediated antigen presentation within the MLN. Cytoplasmatic peptides are not loaded to MHC-II molecules, thus a mechanism dependent on trogocytosis and transfer of peptide-MHC-II complexes has been proposed to explain the induction of Foxp3$^+$ T_{REG} (239). Furthermore, the gel-forming mucins MUC2 activate a β-catenin-dependent tolerogenic pathway in the intestine by binding a Galectin3–Dectin1–FcgRIIB

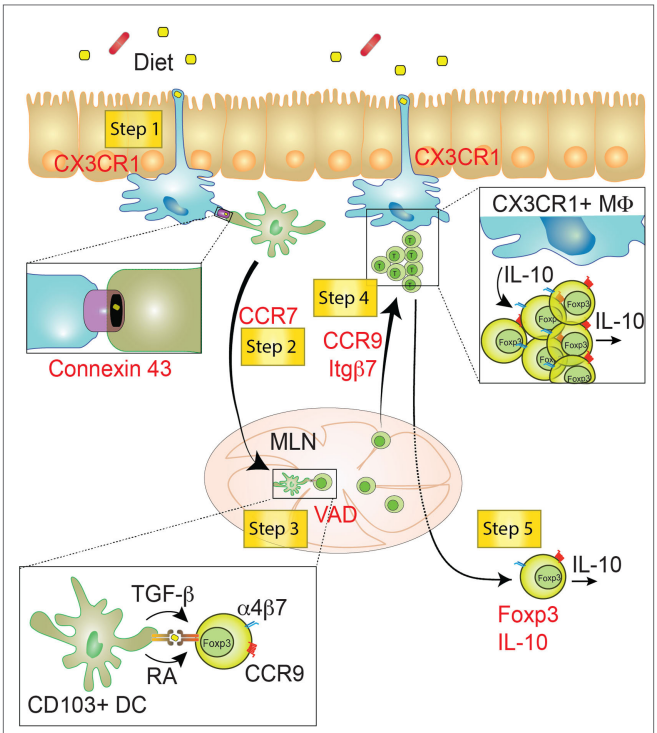

FIGURE 4 | Step-wise induction of oral tolerance. Five different steps can be characterized during the induction of oral tolerance (yellow squares). These steps have been identified using key deficiencies (red), such as genes or vitamins. *Step 1*: CX3CR1$^+$ macrophages (Myeloid cells depicted in blue) might sample food antigens though dendrites crossing the IEC barrier. Food antigens may then be transferred via Connexin 43$^+$ gap junctions from macrophages to migratory CD103$^+$ DCs (Myeloid cells depicted in green). *Step 2:* Food antigen-bearing CD103$^+$ DCs migrate to the MLN in a CCR7-dependent manner and mediate the priming of Foxp3$^+$ T_{REG} through the production of TGF-β and retinoic acid (RA). *Step 3:* newly generated T_{REG} are endowed with the ability to migrate to the small intestine lamina propria. This process relies on the induction of gut-homing receptors (CCR9 and α4β7) in a Vitamin A-dependent manner. *Step 4:* T_{REG} cells migrate to the SI-LP, where they proliferate and acquire tolerogenic properties, likely involving IL-10 produced by CX3CR1$^+$ macrophages. *Step 5:* fully tolerogenic T_{REG} generated in the SI-LP may be released in the circulation preventing peripheral inflammation in a Foxp3- and IL-10-dependent manner. VAD, Vitamin A deficient diet; MLN, mesenteric lymph node; SI-LP, small intestine lamina propria; Itgβ7, integrin β7; MΦ, macrophage.

receptor complex on intestinal CD103$^+$ DCs. Therefore, mice deficient for MUC2 are no longer able to establish intestinal and systemic tolerance upon oral intake of antigens, implying a crucial immunoregulatory role for the mucus in addition to a mere barrier function (240).

Migration to the MLN and Ag Presentation

Following antigen uptake, DCs need to migrate to intestinal draining lymphoid structures in order to present the antigen to cognate naïve T cells and induce antigen-specific Foxp3$^+$ T_{REG}. This has been proven using CCR7$^{-/-}$ mice as well as mesenteric lymphadenectomy, which result in inhibition of oral tolerance induction (241). Unlike the CD103$^-$ subset, migratory CD103$^+$ DCs are endowed with enhanced ability to induce Foxp3$^+$ T_{REG} in a TGF-β and RA-dependent manner (7). Wnt–β-catenin signaling in DCs was shown to increase the expression of regulatory cytokines (*Il10* and *Tgfb1*) and the enzymes responsible for RA production (*Raldh1* and *Raldh2*) (242). CD103$^+$ DC can also be distinguished by expressing the integrin αvβ8, which converts latent to active TGF-β, hence, further specializing them to induce Foxp3$^+$ T_{REG} (10). Thus, CD103$^+$ DC might play a critical role during the establishment of oral tolerance, as suggested by eliminating RA-producing DC precursors expressing α4β7 in the BM, which result in defective Foxp3$^+$ T_{REG} induction (179). Interestingly, among BM hematopoietic cells, other precursors are characterized by the expression of α4β7, including ILC progenitors (31). Hence, it is tempting to speculate that RA regulates ILC homing to the intestine and the consequent induction of oral tolerance. Indeed, a role for intestinal ILCs has recently been proposed in the establishment of tolerance toward food antigens. LTi and NKp46$^+$ ILC3s production of GM-CSF was shown to be essential for the maintenance of resident DCs and for their ability to synthetize RA and produce TGFβ. Noteworthy, GM-CSF production by RORγt$^+$ ILCs was dependent on Mφ sensing microbial signals and subsequent production of IL-1β (243), implying a role for the microbiota in this process. Furthermore, monocolonization with Clostridia confers allergy protection by inducing ILC3-mediated production of IL-22 and by regulating allergens access through the epithelial cell layer (244).

Generation of T_{REG} with Gut Tropism

Interestingly, CD103$^+$ DC also induces the expression of gut-homing receptors, namely CCR9 and α4β7, conferring small intestine tropism to newly generated Foxp3$^+$ T_{REG} (7). In agreement with the role of RA in inducing gut-homing tropism, mice fed with a VAD are impaired in the establishment of oral tolerance. However, the proportion of intestinal Foxp3$^+$ T_{REG} found in VAD and control mice is comparable, suggesting that RA plays a pivotal role in the induction of gut tropism on T cells rather than in the generation of Foxp3$^+$ T_{REG} (5).

Migration of T_{REG} to SI-LP

After the generation of Foxp3$^+$ T_{REG} within the MLN, a previously unpredicted additional step has been recently described: migration to the small intestine lamina propria (SI-LP) rather than to the periphery. The observation that RA induces the expression of α4β7 and CCR9 on Foxp3$^+$ T_{REG}, prompted two different groups to speculate that newly generated Foxp3$^+$ T_{REG} are required to migrate to the small bowel in order to establish oral tolerance. Indeed, mice lacking either CCR9 or integrin β7 showed impaired induction of oral tolerance, which was due to the inability of Foxp3$^+$ T_{REG} to migrate to the proximal small bowel (8, 9). Foxp3$^+$ T_{REG} strongly proliferate in the intestinal lamina propria and most likely they sense CX_3CR1^+ macrophage-derived IL-10, which have been proposed to be key processes required during the establishment of oral tolerance (5, 9). Interestingly, Pabst and colleagues showed that CX_3CR1^+ deficiency in resident intestinal Mφ abrogates oral tolerance without affecting antigen uptake and presentation, but rather impairing IL-10 production and Foxp3$^+$

T_{REG} proliferation in the lamina propria (9). Thus, migration of activated Foxp3$^+$ T_{REG} to the small bowel lamina propria seems to be crucial to fully develop a tolerogenic potential. This is in agreement with a growing body of literature proposing the intestine as a site of education or reprograming even for pathogenic T cells (245). In support of this theory, CCR6-deficient Th17 cells, which are impaired to home to the intestine, possess higher pro-inflammatory properties compared to wild type Th17 (245). Moreover, EAE-induced mice (a mouse model for multiple sclerosis) treated with monoclonal anti-CD3 antibodies are protected due to an increased migration of MOG-specific Th17 cells to the duodenum and reduced Th17 cells in the central nervous system (245). In addition, lung-derived pro-inflammatory cells from mice infected with influenza accumulate in the small intestine where they mediate gastrointestinal symptoms (246). Although the authors did not demonstrate that blocking this migratory pattern could affect the disease in the lungs, it might be possible that influenza-induced gastrointestinal inflammation reflects the cost of reducing inflammation in the lungs and resolving viral infection. Overall, oral tolerance relies on the induction of gut-tropic Foxp3$^+$ T_{REG} specific for fed antigens.

Failure to prompt an effective regulatory response is associated with development of food allergy characterized, predominantly, by a Th2 response. For example, mice carrying a mutation in the IL-4Rα chain that enhances the IL-4R signaling pathway are more prone to develop allergic sensitization to oral antigens, which is associated with Foxp3$^+$ T_{REG} reprogramming toward a Th2 cell-like phenotype (247). Moreover, polymorphisms in the IL4RA locus have been linked to the pathogenesis of human food allergy, and analogously, reprogramed cells (Foxp3$^+$ T_{REG} → Th2) were also found in the peripheral blood of children with food allergy (247).

Deletion and Anergy

A possible alternative step has been proposed for the induction of oral tolerance, involving deletion or anergy induction rather than the generation of regulatory T cells. These different outcomes appear to depend on the different doses of antigens orally administered. In particular, feeding low doses of antigens results in the differentiation of regulatory T cells (as described earlier). Although the most characterized subtype of regulatory T cells is CD4$^+$ Foxp3$^+$ peripherally induced T_{REG}, a role for Tr1 Foxp3$^-$ IL-10-producing T_{REG} and Th3 Foxp3$^-$ LAP$^+$ TGF-β-producing T_{REG} has been described in the low-dose induction of OT. *In vivo,* treatment with anti-LAP mAb was recently shown to abrogate anti-CD3-induced OT in mice (248). Conversely, administration of high doses and thus systemic spreading of the antigen relies on the induction of apoptosis or anergy in effector cells. High-dose feeding with myelin basic protein (MBP) in mice, transgenic for MBP-specific TCR, induces an initial wave of activation of CD4$^+$ T cells and TCR downregulation, followed by anergy and consequent deletion (249). The molecular mechanism underlying the induction of anergy relies on an active process that involves the activation of anergy-associated genes. Ca^{++}/Calcineurin signaling promotes the activation of NFAT that imposes an anergic program if prevented to bind its transcriptional partner AP-1 (250). Among genes that are upregulated on anergic T cells, GRAIL, an E3 ubiquitin ligase, is crucial for the induction of OT. GRAIL$^{-/-}$ mice, genetically engineered to express a TCR specific for the MHC-II restricted peptide of ovalbumin protein (OT-II) on CD4$^+$ T cells, are prevented to establish tolerance to fed OVA antigen (251). Deletion of effector cells is important to avoid reactions against food antigens. Oral gavage of high doses of allergen, in a mouse model of allergic contact dermatitis, promotes plasmacytoid DCs-mediated deletion of Ag-specific CD8$^+$ T cells in the liver and in the MLN. Residual effector CD8$^+$ T cells, that have escaped this first round of tolerance induction, are then subjected to suppression by activated T_{REG} cells, generated as described earlier, in the mucosal associated lymphoid tissues (252).

Most of the studies reported above, aimed at defining OT mechanisms are conducted exploiting TCR-transgenic mouse models that facilitate the tracking of Ag-specific T cells *in vivo*. A general concern about the use of these models is that they are far from being physiologic, since TCR-transgenic T cells recognize their specific antigen with high avidity and generally outnumber the normal repertoire of TCRs specificities. In particular, this might have an impact on studies focused on the induction of anergy and deletion that are influenced by the strength of TCR engagement and signaling.

Concluding Remarks

In order to exert all its biological functions, our body demands energetic fuel that is mainly provided by nutrition. The first diet is represented by breast milk, which is subsequently replaced by solid food intake. It is well accepted that orally delivered nutrients exert a profound biological role in shaping our intestinal immune system, with consequences affecting our susceptibility to gastrointestinal and extra-intestinal disorders. Choosing the diet-regimen in all the stages of our life, starting from breast-feeding (or formula) to the solid diet later on, may have immunological implications, which are not completely understood.

For instance, breast milk-mediated transfer of maternal immune-regulating factors assists the development of the immature immune system of the infant, and help in the protection against the first external pathogenic insults. Numerous breast milk components have been described so far, however, studies aimed at dissecting the molecular and cellular mechanisms involved in the fitness of the immune system, including immune maturation and induction of tolerance, are needed.

In contrast, solid food intake is no longer characterized by the presence of preformed immune mediators, but instead dietary metabolites are endowed with immune-shaping properties that influence the composition of a mature immune system. Several immune effects of food components have been characterized in the last few decades. Even though genetically engineered mice and dietary regimens deprived or supplemented with a specific food metabolite in mouse models have been instrumental, we urgently need to corroborate these findings in humans, to then translate and design novel therapeutic approaches. Observational

and prospective studies in humans may also have an impact on our knowledge on the preventive and therapeutic potential of food. Thus, providing mechanistic insights into how the immune system can be shaped by dietary compounds or breast milk components will offer valuable tools to develop therapeutic strategies against inflammatory disorders or food allergies.

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References

1. Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol* (2014) **14**(3):141–53. doi:10.1038/nri3608
2. Ishifune C, Maruyama S, Sasaki Y, Yagita H, Hozumi K, Tomita T, et al. Differentiation of CD11c+ CX3CR1+ cells in the small intestine requires Notch signaling. *Proc Natl Acad Sci U S A* (2014) **111**(16):5986–91. doi:10.1073/pnas.1401671111
3. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* (2013) **31**:563–604. doi:10.1146/annurev-immunol-020711-074950
4. Schulz O, Jaensson E, Persson EK, Liu X, Worbs T, Agace WW, et al. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med* (2009) **206**(13):3101–14. doi:10.1084/jem.20091925
5. Villablanca EJ. Retinoic acid-producing DCs and gut-tropic FOXP3 regulatory T cells in the induction of oral tolerance. *Oncoimmunology* (2013) **2**(2):e22987. doi:10.4161/onci.22987
6. Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, et al. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J Exp Med* (2008) **205**(9):2139–49. doi:10.1084/jem.20080414
7. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* (2007) **204**(8):1757–64. doi:10.1084/jem.20070590
8. Cassani B, Villablanca EJ, Quintana FJ, Love PE, Lacy-Hulbert A, Blaner WS, et al. Gut-tropic T cells that express integrin alpha4beta7 and CCR9 are required for induction of oral immune tolerance in mice. *Gastroenterology* (2011) **141**(6):2109–18. doi:10.1053/j.gastro.2011.09.015
9. Hadis U, Wahl B, Schulz O, Hardtke-Wolenski M, Schippers A, Wagner N, et al. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity* (2011) **34**(2):237–46. doi:10.1016/j.immuni.2011.01.016
10. Paidassi H, Acharya M, Zhang A, Mukhopadhyay S, Kwon M, Chow C, et al. Preferential expression of integrin alphavbeta8 promotes generation of regulatory T cells by mouse CD103+ dendritic cells. *Gastroenterology* (2011) **141**(5):1813–20. doi:10.1053/j.gastro.2011.06.076
11. Lacy-Hulbert A, Smith AM, Tissire H, Barry M, Crowley D, Bronson RT, et al. Ulcerative colitis and autoimmunity induced by loss of myeloid alphav integrins. *Proc Natl Acad Sci U S A* (2007) **104**(40):15823–8. doi:10.1073/pnas.0707421104
12. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* (2005) **307**(5707):254–8. doi:10.1126/science.1102901
13. Zigmond E, Bernshtein B, Friedlander G, Walker CR, Yona S, Kim KW, et al. Macrophage-restricted interleukin-10 receptor deficiency, but not IL-10 deficiency, causes severe spontaneous colitis. *Immunity* (2014) **40**(5):720–33. doi:10.1016/j.immuni.2014.03.012
14. Shouval DS, Biswas A, Goettel JA, McCann K, Conaway E, Redhu NS, et al. Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity* (2014) **40**(5):706–19. doi:10.1016/j.immuni.2014.03.011
15. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Rosemblatt M, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* (2003) **424**(6944):88–93. doi:10.1038/nature01726
16. Mowat AM, Agace WW. Regional specialization within the intestinal immune system. *Nat Rev Immunol* (2014) **14**(10):667–85. doi:10.1038/nri3738
17. Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* (2003) **3**(4):331–41. doi:10.1038/nri1057
18. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* (2001) **27**(1):20–1. doi:10.1038/83713
19. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* (2001) **27**(1):18–20. doi:10.1038/83707
20. Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, et al. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J Exp Med* (2007) **204**(1):57–63. doi:10.1084/jem.20061852
21. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med* (2013) **19**(6):739–46. doi:10.1038/nm.3179
22. Hawrylowicz CM. Regulatory T cells and IL-10 in allergic inflammation. *J Exp Med* (2005) **202**(11):1459–63. doi:10.1084/jem.20052211
23. Gasteiger G, Hemmers S, Bos PD, Sun JC, Rudensky AY. IL-2-dependent adaptive control of NK cell homeostasis. *J Exp Med* (2013) **210**(6):1179–87. doi:10.1084/jem.20122571
24. Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity* (2012) **37**(4):649–59. doi:10.1016/j.immuni.2012.08.015
25. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* (2008) **8**(7):523–32. doi:10.1038/nri2343
26. Bartlett HS, Million RP. Targeting the IL-17-T(H)17 pathway. *Nat Rev Drug Discov* (2015) **14**(1):11–2. doi:10.1038/nrd4518
27. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* (2007) **8**(9):942–9. doi:10.1038/ni1496
28. Huber S, Gagliani N, Flavell RA. Life, death, and miracles: Th17 cells in the intestine. *Eur J Immunol* (2012) **42**(9):2238–45. doi:10.1002/eji.201242619
29. Gagliani N, Vesely MC, Iseppon A, Brockmann L, Xu H, Palm NW, et al. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. *Nature* (2015) **523**(7559):221–5. doi:10.1038/nature14452
30. Walker JA, Barlow JL, McKenzie AN. Innate lymphoid cells – how did we miss them? *Nat Rev Immunol* (2013) **13**(2):75–87. doi:10.1038/nri3349
31. Klose CS, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell* (2014) **157**(2):340–56. doi:10.1016/j.cell.2014.03.030
32. Constantinides MG, McDonald BD, Verhoef PA, Bendelac A. A committed precursor to innate lymphoid cells. *Nature* (2014) **508**(7496):397–401. doi:10.1038/nature13047
33. Constantinides MG, Gudjonson H, McDonald BD, Ishizuka IE, Verhoef PA, Dinner AR, et al. PLZF expression maps the early stages of ILC1 lineage development. *Proc Natl Acad Sci U S A* (2015) **112**(16):5123–8. doi:10.1073/pnas.1423244112
34. Cording S, Medvedovic J, Cherrier M, Eberl G. Development and regulation of RORgammat innate lymphoid cells. *FEBS Lett* (2014) **588**(22):4176–81. doi:10.1016/j.febslet.2014.03.034
35. Coccia M, Harrison OJ, Schiering C, Asquith MJ, Becher B, Powrie F, et al. IL-1beta mediates chronic intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4(+) Th17 cells. *J Exp Med* (2012) **209**(9):1595–609. doi:10.1084/jem.20111453

36. Buonocore S, Ahern PP, Uhlig HH, Ivanov II, Littman DR, Maloy KJ, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature* (2010) **464**(7293):1371–5. doi:10.1038/nature08949
37. Powell N, Walker AW, Stolarczyk E, Canavan JB, Gokmen MR, Marks E, et al. The transcription factor T-bet regulates intestinal inflammation mediated by interleukin-7 receptor+ innate lymphoid cells. *Immunity* (2012) **37**(4):674–84. doi:10.1016/j.immuni.2012.09.008
38. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* (2012) **491**(7422):119–24. doi:10.1038/nature11582
39. Satoh-Takayama N, Vosshenrich CA, Lesjean-Pottier S, Sawa S, Lochner M, Rattis F, et al. Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity* (2008) **29**(6):958–70. doi:10.1016/j.immuni.2008.11.001
40. Sonnenberg GF, Monticelli LA, Alenghat T, Fung TC, Hutnick NA, Kunisawa J, et al. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science* (2012) **336**(6086):1321–5. doi:10.1126/science.1222551
41. Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med* (2008) **14**(3):282–9. doi:10.1038/nm1720
42. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. *Immunity* (2008) **29**(6):947–57. doi:10.1016/j.immuni.2008.11.003
43. Mackley EC, Houston S, Marriott CL, Halford EE, Lucas B, Cerovic V, et al. CCR7-dependent trafficking of RORgamma(+) ILCs creates a unique microenvironment within mucosal draining lymph nodes. *Nat Commun* (2015) **6**:5862. doi:10.1038/ncomms6862
44. Hepworth MR, Monticelli LA, Fung TC, Ziegler CG, Grunberg S, Sinha R, et al. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. *Nature* (2013) **498**(7452):113–7. doi:10.1038/nature12240
45. Hepworth MR, Fung TC, Masur SH, Kelsen JR, McConnell FM, Dubrot J, et al. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4+ T cells. *Science* (2015) **348**(6238):1031–5. doi:10.1126/science.aaa4812
46. Oliphant CJ, Hwang YY, Walker JA, Salimi M, Wong SH, Brewer JM, et al. MHCII-mediated dialog between group 2 innate lymphoid cells and CD4(+) T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. *Immunity* (2014) **41**(2):283–95. doi:10.1016/j.immuni.2014.06.016
47. Gasteiger G, Rudensky AY. Interactions between innate and adaptive lymphocytes. *Nat Rev Immunol* (2014) **14**(9):631–9. doi:10.1038/nri3726
48. Ivanov II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* (2008) **4**(4):337–49. doi:10.1016/j.chom.2008.09.009
49. Atarashi K, Nishimura J, Shima T, Umesaki Y, Yamamoto M, Onoue M, et al. ATP drives lamina propria T(H)17 cell differentiation. *Nature* (2008) **455**(7214):808–12. doi:10.1038/nature07240
50. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* (2009) **9**(5):313–23. doi:10.1038/nri2515
51. M'Rabet L, Vos AP, Boehm G, Garssen J. Breast-feeding and its role in early development of the immune system in infants: consequences for health later in life. *J Nutr* (2008) **138**(9):1782S–90S.
52. Munblit D, Boyle RJ. Modulating breast milk composition – the key to allergy prevention? *Int Arch Allergy Immunol* (2012) **159**(2):107–8. doi:10.1159/000338192
53. Yngve A, Sjostrom M. Breastfeeding determinants and a suggested framework for action in Europe. *Public Health Nutr* (2001) **4**(2B):729–39. doi:10.1079/PHN2001164
54. Tariq SM, Matthews SM, Hakim EA, Stevens M, Arshad SH, Hide DW. The prevalence of and risk factors for atopy in early childhood: a whole population birth cohort study. *J Allergy Clin Immunol* (1998) **101**(5):587–93. doi:10.1016/S0091-6749(98)70164-2
55. Oddy WH, Holt PG, Sly PD, Read AW, Landau LI, Stanley FJ, et al. Association between breast feeding and asthma in 6 year old children: findings of a prospective birth cohort study. *BMJ* (1999) **319**(7213):815–9. doi:10.1136/bmj.319.7213.815
56. Giwercman C, Halkjaer LB, Jensen SM, Bonnelykke K, Lauritzen L, Bisgaard H. Increased risk of eczema but reduced risk of early wheezy disorder from exclusive breast-feeding in high-risk infants. *J Allergy Clin Immunol* (2010) **125**(4):866–71. doi:10.1016/j.jaci.2010.01.026
57. van Odijk J, Kull I, Borres MP, Brandtzaeg P, Edberg U, Hanson LA, et al. Breastfeeding and allergic disease: a multidisciplinary review of the literature (1966-2001) on the mode of early feeding in infancy and its impact on later atopic manifestations. *Allergy* (2003) **58**(9):833–43. doi:10.1034/j.1398-9995.2003.00264.x
58. Knip M, Virtanen SM, Akerblom HK. Infant feeding and the risk of type 1 diabetes. *Am J Clin Nutr* (2010) **91**(5):1506S–13S. doi:10.3945/ajcn.2010.28701C
59. Lund-Blix NA, Stene LC, Rasmussen T, Torjesen PA, Andersen LF, Ronningen KS. Infant feeding in relation to islet autoimmunity and type 1 diabetes in genetically susceptible children: the MIDIA Study. *Diabetes Care* (2015) **38**(2):257–63. doi:10.2337/dc14-1130
60. Klement E, Cohen RV, Boxman J, Joseph A, Reif S. Breastfeeding and risk of inflammatory bowel disease: a systematic review with meta-analysis. *Am J Clin Nutr* (2004) **80**(5):1342–52.
61. Conradi S, Malzahn U, Paul F, Quill S, Harms L, Then Bergh F, et al. Breastfeeding is associated with lower risk for multiple sclerosis. *Mult Scler* (2013) **19**(5):553–8. doi:10.1177/1352458512459683
62. McManaman JL, Neville MC. Mammary physiology and milk secretion. *Adv Drug Deliv Rev* (2003) **55**(5):629–41. doi:10.1016/S0169-409X(03)00033-4
63. Saint L, Smith M, Hartmann PE. The yield and nutrient content of colostrum and milk of women from giving birth to 1 month post-partum. *Br J Nutr* (1984) **52**(1):87–95. doi:10.1079/BJN19840074
64. Pang WW, Hartmann PE. Initiation of human lactation: secretory differentiation and secretory activation. *J Mammary Gland Biol Neoplasia* (2007) **12**(4):211–21. doi:10.1007/s10911-007-9054-4
65. Kulski JK, Hartmann PE. Changes in human milk composition during the initiation of lactation. *Aust J Exp Biol Med Sci* (1981) **59**(1):101–14. doi:10.1038/icb.1981.66
66. Castellote C, Casillas R, Ramirez-Santana C, Perez-Cano FJ, Castell M, Moretones MG, et al. Premature delivery influences the immunological composition of colostrum and transitional and mature human milk. *J Nutr* (2011) **141**(6):1181–7. doi:10.3945/jn.110.133652
67. Kaetzel CS. The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces. *Immunol Rev* (2005) **206**:83–99. doi:10.1111/j.0105-2896.2005.00278.x
68. Van de Perre P. Transfer of antibody via mother's milk. *Vaccine* (2003) **21**(24):3374–6. doi:10.1016/S0264-410X(03)00336-0
69. Hanson LA. Breastfeeding provides passive and likely long-lasting active immunity. *Ann Allergy Asthma Immunol* (1998) **81**(6):523–33. doi:10.1016/S1081-1206(10)62704-4
70. Selner JC, Merrill DA, Claman HN. Salivary immunoglobulin and albumin: development during the newborn period. *J Pediatr* (1968) **72**(5):685–9. doi:10.1016/S0022-3476(68)80014-9
71. McLean B, Holmes IH. Transfer of antirotaviral antibodies from mothers to their infants. *J Clin Microbiol* (1980) **12**(3):320–5.
72. Stoliar OA, Pelley RP, Kaniecki-Green E, Kkaus MH, Carpenter CC. Secretory IgA against enterotoxins in breast-milk. *Lancet* (1976) **1**(7972):1258–61. doi:10.1016/S0140-6736(76)91735-9
73. Rogier EW, Frantz AL, Bruno ME, Wedlund L, Cohen DA, Stromberg AJ, et al. Secretory antibodies in breast milk promote long-term intestinal homeostasis by regulating the gut microbiota and host gene expression. *Proc Natl Acad Sci U S A* (2014) **111**(8):3074–9. doi:10.1073/pnas.1315792111
74. Kukkonen K, Kuitunen M, Haahtela T, Korpela R, Poussa T, Savilahti E. High intestinal IgA associates with reduced risk of IgE-associated allergic diseases. *Pediatr Allergy Immunol* (2010) **21**(1 Pt 1):67–73. doi:10.1111/j.1399-3038.2009.00907.x
75. Uthoff H, Spenner A, Reckelkamm W, Ahrens B, Wolk G, Hackler R, et al. Critical role of preconceptional immunization for protective and nonpathological specific immunity in murine neonates. *J Immunol* (2003) **171**(7):3485–92. doi:10.4049/jimmunol.171.7.3485

76. Hochwallner H, Alm J, Lupinek C, Johansson C, Mie A, Scheynius A, et al. Transmission of allergen-specific IgG and IgE from maternal blood into breast milk visualized with microarray technology. *J Allergy Clin Immunol* (2014) **134**(5):1213–5. doi:10.1016/j.jaci.2014.08.041
77. Matson AP, Thrall RS, Rafti E, Puddington L. Breastmilk from allergic mothers can protect offspring from allergic airway inflammation. *Breastfeed Med* (2009) **4**(3):167–74. doi:10.1089/bfm.2008.0130
78. Shimamura M, Huang YY, Goji H. Antibody production in early life supported by maternal lymphocyte factors. *Biochim Biophys Acta* (2003) **1637**(1):55–8. doi:10.1016/S0925-4439(02)00211-9
79. Dutta P, Molitor-Dart M, Bobadilla JL, Roenneburg DA, Yan Z, Torrealba JR, et al. Microchimerism is strongly correlated with tolerance to noninherited maternal antigens in mice. *Blood* (2009) **114**(17):3578–87. doi:10.1182/blood-2009-03-213561
80. Evans RS, Pestotnik SL, Burke JP, Gardner RM, Larsen RA, Classen DC. Reducing the duration of prophylactic antibiotic use through computer monitoring of surgical patients. *DICP* (1990) **24**(4):351–4.
81. Hassiotou F, Hepworth AR, Metzger P, Tat Lai C, Trengove N, Hartmann PE, et al. Maternal and infant infections stimulate a rapid leukocyte response in breastmilk. *Clin Transl Immunology* (2013) **2**(4):e3. doi:10.1038/cti.2013.1
82. Ho FC, Wong RL, Lawton JW. Human colostral and breast milk cells. A light and electron microscopic study. *Acta Paediatr Scand* (1979) **68**(3):389–96. doi:10.1111/j.1651-2227.1979.tb05025.x
83. Brooker BE. The epithelial cells and cell fragments in human milk. *Cell Tissue Res* (1980) **210**(2):321–32. doi:10.1007/BF00237619
84. Goldman AS, Goldblum RM. Transfer of maternal leukocytes to the infant by human milk. *Curr Top Microbiol Immunol* (1997) **222**:205–13.
85. Xanthou M. Immune protection of human milk. *Biol Neonate* (1998) **74**(2):121–33. doi:10.1159/000014018
86. Lea PJ, Pawlowski A. Human melanocytic naevi. II. Depth of dermal protrusion: comparative measurements of the basement membrane zone and diameters of intracellular microfibrils using a microcomputer analysis system. *Acta Derm Venereol Suppl* (1986) **127**:17–21.
87. Weiler IJ, Hickler W, Sprenger R. Demonstration that milk cells invade the suckling neonatal mouse. *Am J Reprod Immunol* (1983) **4**(2):95–8. doi:10.1111/j.1600-0897.1983.tb00261.x
88. Sheldrake RF, Husband AJ. Intestinal uptake of intact maternal lymphocytes by neonatal rats and lambs. *Res Vet Sci* (1985) **39**(1):10–5.
89. Jain L, Vidyasagar D, Xanthou M, Ghai V, Shimada S, Blend M. In vivo distribution of human milk leucocytes after ingestion by newborn baboons. *Arch Dis Child* (1989) **64**(7 Spec No):930–3. doi:10.1136/adc.64.7_Spec_No.930
90. Rivas RA, el-Mohandes AA, Katona IM. Mononuclear phagocytic cells in human milk: HLA-DR and Fc gamma R ligand expression. *Biol Neonate* (1994) **66**(4):195–204. doi:10.1159/000244108
91. Keeney SE, Schmalstieg FC, Palkowetz KH, Rudloff HE, Le BM, Goldman AS. Activated neutrophils and neutrophil activators in human milk: increased expression of CD11b and decreased expression of L-selectin. *J Leukoc Biol* (1993) **54**(2):97–104.
92. Tsiang H, Atanasiu P, Chermann JC, Jasmin C. Inhibition of rabies virus in vitro by the ammonium-5-tungsto-2-antimoniate. *J Gen Virol* (1978) **40**(3):665–8. doi:10.1099/0022-1317-40-3-665
93. Lindstrand A, Smedman L, Gunnlaugsson G, Troye-Blomberg M. Selective compartmentalization of gammadelta-T lymphocytes in human breastmilk. *Acta Paediatr* (1997) **86**(8):890–1. doi:10.1111/j.1651-2227.1997.tb08617.x
94. Kourtis AP, Ibegbu CC, Theiler R, Xu YX, Bansil P, Jamieson DJ, et al. Breast milk CD4+ T cells express high levels of C chemokine receptor 5 and CXC chemokine receptor 4 and are preserved in HIV-infected mothers receiving highly active antiretroviral therapy. *J Infect Dis* (2007) **195**(7):965–72. doi:10.1086/512082
95. Sabbaj S, Ghosh MK, Edwards BH, Leeth R, Decker WD, Goepfert PA, et al. Breast milk-derived antigen-specific CD8+ T cells: an extralymphoid effector memory cell population in humans. *J Immunol* (2005) **174**(5):2951–6. doi:10.4049/jimmunol.174.5.2951
96. Bertotto A, Castellucci G, Fabietti G, Scalise F, Vaccaro R. Lymphocytes bearing the T cell receptor gamma delta in human breast milk. *Arch Dis Child* (1990) **65**(11):1274–5. doi:10.1136/adc.65.11.1274-a
97. Peroni DG, Chirumbolo S, Veneri D, Piacentini GL, Tenero L, Vella A, et al. Colostrum-derived B and T cells as an extra-lymphoid compartment of effector cell populations in humans. *J Matern Fetal Neonatal Med* (2013) **26**(2):137–42. doi:10.3109/14767058.2012.733744
98. Tuaillon E, Valea D, Becquart P, Al Tabaa Y, Meda N, Bollore K, et al. Human milk-derived B cells: a highly activated switched memory cell population primed to secrete antibodies. *J Immunol* (2009) **182**(11):7155–62. doi:10.4049/jimmunol.0803107
99. Brandtzaeg P. Mucosal immunity: integration between mother and the breast-fed infant. *Vaccine* (2003) **21**(24):3382–8. doi:10.1016/S0264-410X(03)00338-4
100. Wilson E, Butcher EC. CCL28 controls immunoglobulin (Ig)A plasma cell accumulation in the lactating mammary gland and IgA antibody transfer to the neonate. *J Exp Med* (2004) **200**(6):805–9. doi:10.1084/jem.20041069
101. Palmer DJ, Gold MS, Makrides M. Effect of cooked and raw egg consumption on ovalbumin content of human milk: a randomized, double-blind, cross-over trial. *Clin Exp Allergy* (2005) **35**(2):173–8. doi:10.1111/j.1365-2222.2005.02170.x
102. Jarvinen KM, Westfall JE, Seppo MS, James AK, Tsuang AJ, Feustel PJ, et al. Role of maternal elimination diets and human milk IgA in the development of cow's milk allergy in the infants. *Clin Exp Allergy* (2014) **44**(1):69–78. doi:10.1111/cea.12228
103. Bernard H, Ah-Leung S, Drumare MF, Feraudet-Tarisse C, Verhasselt V, Wal JM, et al. Peanut allergens are rapidly transferred in human breast milk and can prevent sensitization in mice. *Allergy* (2014) **69**(7):888–97. doi:10.1111/all.12411
104. Tooley KL, El-Merhibi A, Cummins AG, Grose RH, Lymn KA, DeNichilo M, et al. Maternal milk, but not formula, regulates the immune response to beta-lactoglobulin in allergy-prone rat pups. *J Nutr* (2009) **139**(11):2145–51. doi:10.3945/jn.109.108845
105. Yamamoto T, Tsubota Y, Kodama T, Kageyama-Yahara N, Kadowaki M. Oral tolerance induced by transfer of food antigens via breast milk of allergic mothers prevents offspring from developing allergic symptoms in a mouse food allergy model. *Clin Dev Immunol* (2012) **2012**:721085. doi:10.1155/2012/721085
106. Macchiaverni P, Rekima A, Turfkruyer M, Mascarell L, Airouche S, Moingeon P, et al. Respiratory allergen from house dust mite is present in human milk and primes for allergic sensitization in a mouse model of asthma. *Allergy* (2014) **69**(3):395–8. doi:10.1111/all.12332
107. Chua KY, Stewart GA, Thomas WR, Simpson RJ, Dilworth RJ, Plozza TM, et al. Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. *J Exp Med* (1988) **167**(1):175–82. doi:10.1084/jem.167.1.175
108. Verhasselt V, Milcent V, Cazareth J, Kanda A, Fleury S, Dombrowicz D, et al. Breast milk-mediated transfer of an antigen induces tolerance and protection from allergic asthma. *Nat Med* (2008) **14**(2):170–5. doi:10.1038/nm1718
109. Garofalo R. Cytokines in human milk. *J Pediatr* (2010) **156**(2 Suppl):S36–40. doi:10.1016/j.jpeds.2009.11.019
110. Bottcher MF, Jenmalm MC, Garofalo RP, Bjorksten B. Cytokines in breast milk from allergic and nonallergic mothers. *Pediatr Res* (2000) **47**(1):157–62. doi:10.1203/00006450-200005000-00006
111. Saito S, Maruyama M, Kato Y, Moriyama I, Ichijo M. Detection of IL-6 in human milk and its involvement in IgA production. *J Reprod Immunol* (1991) **20**(3):267–76. doi:10.1016/0165-0378(91)90051-Q
112. Bottcher MF, Jenmalm MC, Bjorksten B, Garofalo RP. Chemoattractant factors in breast milk from allergic and nonallergic mothers. *Pediatr Res* (2000) **47**(5):592–7. doi:10.1203/00006450-200005000-00006
113. Garofalo R, Chheda S, Mei F, Palkowetz KH, Rudloff HE, Schmalstieg FC, et al. Interleukin-10 in human milk. *Pediatr Res* (1995) **37**(4 Pt 1):444–9. doi:10.1203/00006450-199504000-00010
114. Grazioso CF, Werner AL, Alling DW, Bishop PR, Buescher ES. Antiinflammatory effects of human milk on chemically induced colitis in rats. *Pediatr Res* (1997) **42**(5):639–43. doi:10.1203/00006450-199711000-00015
115. Ward TL, Spencer WJ, Davis LD, Harrold J, Mack DR, Altosaar I. Ingested soluble CD14 from milk is transferred intact into the blood of newborn rats. *Pediatr Res* (2014) **75**(2):252–8. doi:10.1038/pr.2013.225
116. Labeta MO, Vidal K, Nores JE, Arias M, Vita N, Morgan BP, et al. Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14. *J Exp Med* (2000) **191**(10):1807–12. doi:10.1084/jem.191.10.1807

117. Jones CA, Holloway JA, Popplewell EJ, Diaper ND, Holloway JW, Vance GH, et al. Reduced soluble CD14 levels in amniotic fluid and breast milk are associated with the subsequent development of atopy, eczema, or both. *J Allergy Clin Immunol* (2002) **109**(5):858–66. doi:10.1067/mai.2002.123535
118. Ismail IH, Licciardi PV, Oppedisano F, Boyle RJ, Tang ML. Relationship between breast milk sCD14, TGF-beta1 and total IgA in the first month and development of eczema during infancy. *Pediatr Allergy Immunol* (2013) **24**(4):352–60. doi:10.1111/pai.12075
119. Jagadeesan V, Reddy V. C3 in human milk. *Acta Paediatr Scand* (1978) **67**(2):237–8. doi:10.1111/j.1651-2227.1978.tb16310.x
120. Sakurai T, Furukawa M, Asoh M, Kanno T, Kojima T, Yonekubo A. Fat-soluble and water-soluble vitamin contents of breast milk from Japanese women. *J Nutr Sci Vitaminol* (2005) **51**(4):239–47. doi:10.3177/jnsv.51.239
121. Romeu-Nadal M, Morera-Pons S, Castellote AI, Lopez-Sabater MC. Determination of gamma- and alpha-tocopherols in human milk by a direct high-performance liquid chromatographic method with UV-vis detection and comparison with evaporative light scattering detection. *J Chromatogr A* (2006) **1114**(1):132–7. doi:10.1016/j.chroma.2006.02.049
122. Perez PF, Dore J, Leclerc M, Levenez F, Benyacoub J, Serrant P, et al. Bacterial imprinting of the neonatal immune system: lessons from maternal cells? *Pediatrics* (2007) **119**(3):e724–32. doi:10.1542/peds.2006-1649
123. Jost T, Lacroix C, Braegger CP, Rochat F, Chassard C. Vertical mother-neonate transfer of maternal gut bacteria via breastfeeding. *Environ Microbiol* (2014) **16**(9):2891–904. doi:10.1111/1462-2920.12238
124. Martin R, Langa S, Reviriego C, Jiminez E, Marin ML, Xaus J, et al. Human milk is a source of lactic acid bacteria for the infant gut. *J Pediatr* (2003) **143**(6):754–8. doi:10.1016/j.jpeds.2003.09.028
125. Martin R, Olivares M, Marin ML, Fernandez L, Xaus J, Rodriguez JM. Probiotic potential of 3 lactobacilli strains isolated from breast milk. *J Hum Lact* (2005) **21**(1):8–17. doi:10.1177/0890334404272393
126. Heikkila MP, Saris PE. Inhibition of *Staphylococcus aureus* by the commensal bacteria of human milk. *J Appl Microbiol* (2003) **95**(3):471–8. doi:10.1046/j.1365-2672.2003.02002.x
127. Martin R, Jimenez E, Heilig H, Fernandez L, Marin ML, Zoetendal EG, et al. Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR. *Appl Environ Microbiol* (2009) **75**(4):965–9. doi:10.1128/AEM.02063-08
128. Bertelsen RJ, Brantsaeter AL, Magnus MC, Haugen M, Myhre R, Jacobsson B, et al. Probiotic milk consumption in pregnancy and infancy and subsequent childhood allergic diseases. *J Allergy Clin Immunol* (2014) **133**(1):e1–8. doi:10.1016/j.jaci.2013.07.032
129. Holmgren J, Svennerholm AM, Lindblad M. Receptor-like glycocompounds in human milk that inhibit classical and El Tor *Vibrio cholerae* cell adherence (hemagglutination). *Infect Immun* (1983) **39**(1):147–54.
130. Coppa GV, Zampini L, Galeazzi T, Facinelli B, Ferrante L, Capretti R, et al. Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrheal pathogens: *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris*. *Pediatr Res* (2006) **59**(3):377–82. doi:10.1203/01.pdr.0000200805.45593.17
131. Motley MA, Arnold RR. Cofactor requirements for expression of lactoferrin bactericidal activity on enteric bacteria. *Adv Exp Med Biol* (1987) **216A**:591–9. doi:10.1007/978-1-4684-5344-7_71
132. Petschow BW, Talbott RD. Response of bifidobacterium species to growth promoters in human and cow milk. *Pediatr Res* (1991) **29**(2):208–13. doi:10.1203/00006450-199102000-00021
133. Thormar H, Isaacs CE, Brown HR, Barshatzky MR, Pessolano T. Inactivation of enveloped viruses and killing of cells by fatty acids and monoglycerides. *Antimicrob Agents Chemother* (1987) **31**(1):27–31. doi:10.1128/AAC.31.1.27
134. Chipman DM, Pollock JJ, Sharon N. Lysozyme-catalyzed hydrolysis and transglycosylation reactions of bacterial cell wall oligosaccharides. *J Biol Chem* (1968) **243**(3):487–96.
135. Lee-Huang S, Maiorov V, Huang PL, Ng A, Lee HC, Chang YT, et al. Structural and functional modeling of human lysozyme reveals a unique nonapeptide, HL9, with anti-HIV activity. *Biochemistry* (2005) **44**(12):4648–55. doi:10.1021/bi0477081
136. Mather IH, Keenan TW. Origin and secretion of milk lipids. *J Mammary Gland Biol Neoplasia* (1998) **3**(5):259–73. doi:10.1023/A:1018711410270
137. Peterson JA, Patton S, Hamosh M. Glycoproteins of the human milk fat globule in the protection of the breast-fed infant against infections. *Biol Neonate* (1998) **74**(2):143–62. doi:10.1159/000014020
138. Heid HW, Keenan TW. Intracellular origin and secretion of milk fat globules. *Eur J Cell Biol* (2005) **84**(2–3):245–58. doi:10.1016/j.ejcb.2004.12.002
139. Hamosh M, Peterson JA, Henderson TR, Scallan CD, Kiwan R, Ceriani RL, et al. Protective function of human milk: the milk fat globule. *Semin Perinatol* (1999) **23**(3):242–9. doi:10.1016/S0146-0005(99)80069-X
140. Guang W, Ding H, Czinn SJ, Kim KC, Blanchard TG, Lillehoj EP. Muc1 cell surface mucin attenuates epithelial inflammation in response to a common mucosal pathogen. *J Biol Chem* (2010) **285**(27):20547–57. doi:10.1074/jbc.M110.121319
141. Sheng YH, Triyana S, Wang R, Das I, Gerloff K, Florin TH, et al. MUC1 and MUC13 differentially regulate epithelial inflammation in response to inflammatory and infectious stimuli. *Mucosal Immunol* (2013) **6**(3):557–68. doi:10.1038/mi.2012.98
142. Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, et al. Exosomes with immune modulatory features are present in human breast milk. *J Immunol* (2007) **179**(3):1969–78. doi:10.4049/jimmunol.179.3.1969
143. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. *J Proteomics* (2010) **73**(10):1907–20. doi:10.1016/j.jprot.2010.06.006
144. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* (2007) **9**(6):654–9. doi:10.1038/ncb1596
145. Lasser C, Alikhani VS, Ekstrom K, Eldh M, Paredes PT, Bossios A, et al. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. *J Transl Med* (2011) **9**:9. doi:10.1186/1479-5876-9-9
146. Zhou Q, Li M, Wang X, Li Q, Wang T, Zhu Q, et al. Immune-related microRNAs are abundant in breast milk exosomes. *Int J Biol Sci* (2012) **8**(1):118–23. doi:10.7150/ijbs.8.118
147. Welton JL, Khanna S, Giles PJ, Brennan P, Brewis IA, Staffurth J, et al. Proteomics analysis of bladder cancer exosomes. *Mol Cell Proteomics* (2010) **9**(6):1324–38. doi:10.1074/mcp.M000063-MCP201
148. Segura E, Nicco C, Lombard B, Veron P, Raposo G, Batteux F, et al. ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. *Blood* (2005) **106**(1):216–23. doi:10.1182/blood-2005-01-0220
149. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* (1996) **183**(3):1161–72. doi:10.1084/jem.183.3.1161
150. Karlsson M, Lundin S, Dahlgren U, Kahu H, Pettersson I, Telemo E. "Tolerosomes" are produced by intestinal epithelial cells. *Eur J Immunol* (2001) **31**(10):2892–900. doi:10.1002/1521-4141(2001010)31:10<2892::AID-IMMU2892>3.0.CO;2-I
151. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* (1998) **4**(5):594–600. doi:10.1038/nm0598-594
152. Admyre C, Johansson SM, Paulie S, Gabrielsson S. Direct exosome stimulation of peripheral human T cells detected by ELISPOT. *Eur J Immunol* (2006) **36**(7):1772–81. doi:10.1002/eji.200535615
153. Hwang I, Shen X, Sprent J. Direct stimulation of naive T cells by membrane vesicles from antigen-presenting cells: distinct roles for CD54 and B7 molecules. *Proc Natl Acad Sci U S A* (2003) **100**(11):6670–5. doi:10.1073/pnas.1131852100
154. Hao S, Bai O, Li F, Yuan J, Laferte S, Xiang J. Mature dendritic cells pulsed with exosomes stimulate efficient cytotoxic T-lymphocyte responses and antitumour immunity. *Immunology* (2007) **120**(1):90–102. doi:10.1111/j.1365-2567.2006.02483.x
155. Pieters BC, Arntz OJ, Bennink MB, Broeren MG, van Caam AP, Koenders MI, et al. Commercial cow milk contains physically stable extracellular vesicles expressing immunoregulatory TGF-beta. *PLoS One* (2015) **10**(3):e0121123. doi:10.1371/journal.pone.0121123
156. Sun Q, Chen X, Yu J, Zen K, Zhang CY, Li L. Immune modulatory function of abundant immune-related microRNAs in microvesicles from bovine colostrum. *Protein Cell* (2013) **4**(3):197–210. doi:10.1007/s13238-013-2119-9

157. Prado N, Canamero M, Villalba M, Rodriguez R, Batanero E. Bystander suppression to unrelated allergen sensitization through intranasal administration of tolerogenic exosomes in mouse. *Mol Immunol* (2010) **47**(11–12):2148–51. doi:10.1016/j.molimm.2010.04.014
158. Torregrosa Paredes P, Gutzeit C, Johansson S, Admyre C, Stenius F, Alm J, et al. Differences in exosome populations in human breast milk in relation to allergic sensitization and lifestyle. *Allergy* (2014) **69**(4):463–71. doi:10.1111/all.12357
159. Naslund TI, Paquin-Proulx D, Paredes PT, Vallhov H, Sandberg JK, Gabrielsson S. Exosomes from breast milk inhibit HIV-1 infection of dendritic cells and subsequent viral transfer to CD4+ T cells. *AIDS* (2014) **28**(2):171–80. doi:10.1097/QAD.0000000000000159
160. Backhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* (2015) **17**(5):690–703. doi:10.1016/j.chom.2015.04.004
161. Veldhoen M, Brucklacher-Waldert V. Dietary influences on intestinal immunity. *Nat Rev Immunol* (2012) **12**(10):696–708. doi:10.1038/nri3299
162. Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. *Nat Immunol* (2011) **12**(1):5–9. doi:10.1038/ni0111-5
163. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome and the immune system. *Nature* (2011) **474**(7351):327–36. doi:10.1038/nature10213
164. West KP Jr. Vitamin A deficiency disorders in children and women. *Food Nutr Bull* (2003) **24**(4 Suppl):S78–90.
165. Villamor E, Fawzi WW. Effects of vitamin a supplementation on immune responses and correlation with clinical outcomes. *Clin Microbiol Rev* (2005) **18**(3):446–64. doi:10.1128/CMR.18.3.446-464.2005
166. Hall JA, Grainger JR, Spencer SP, Belkaid Y. The role of retinoic acid in tolerance and immunity. *Immunity* (2011) **35**(1):13–22. doi:10.1016/j.immuni.2011.07.002
167. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* (2004) **21**(4):527–38. doi:10.1016/j.immuni.2004.08.011
168. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, et al. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science* (2006) **314**(5802):1157–60. doi:10.1126/science.1132742
169. Cassani B, Villablanca EJ, De Calisto J, Wang S, Mora JR. Vitamin A and immune regulation: role of retinoic acid in gut-associated dendritic cell education, immune protection and tolerance. *Mol Aspects Med* (2012) **33**(1):63–76. doi:10.1016/j.mam.2011.11.001
170. Brown CC, Esterhazy D, Sarde A, London M, Pullabhatla V, Osma-Garcia I, et al. Retinoic acid is essential for Th1 cell lineage stability and prevents transition to a Th17 cell program. *Immunity* (2015) **42**(3):499–511. doi:10.1016/j.immuni.2015.02.003
171. DePaolo RW, Abadie V, Tang F, Fehlner-Peach H, Hall JA, Wang W, et al. Co-adjuvant effects of retinoic acid and IL-15 induce inflammatory immunity to dietary antigens. *Nature* (2011) **471**(7337):220–4. doi:10.1038/nature09849
172. Cha HR, Chang SY, Chang JH, Kim JO, Yang JY, Kim CH, et al. Downregulation of Th17 cells in the small intestine by disruption of gut flora in the absence of retinoic acid. *J Immunol* (2010) **184**(12):6799–806. doi:10.4049/jimmunol.0902944
173. Spencer SP, Wilhelm C, Yang Q, Hall JA, Bouladoux N, Boyd A, et al. Adaptation of innate lymphoid cells to a micronutrient deficiency promotes type 2 barrier immunity. *Science* (2014) **343**(6169):432–7. doi:10.1126/science.1247606
174. Mielke LA, Jones SA, Raverdeau M, Higgs R, Stefanska A, Groom JR, et al. Retinoic acid expression associates with enhanced IL-22 production by gammadelta T cells and innate lymphoid cells and attenuation of intestinal inflammation. *J Exp Med* (2013) **210**(6):1117–24. doi:10.1084/jem.20121588
175. van de Pavert SA, Ferreira M, Domingues RG, Ribeiro H, Molenaar R, Moreira-Santos L, et al. Maternal retinoids control type 3 innate lymphoid cells and set the offspring immunity. *Nature* (2014) **508**(7494):123–7. doi:10.1038/nature13158
176. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. *Nat Rev Immunol* (2008) **8**(9):685–98. doi:10.1038/nri2378
177. Villablanca EJ, Wang S, de Calisto J, Gomes DC, Kane MA, Napoli JL, et al. MyD88 and retinoic acid signaling pathways interact to modulate gastrointestinal activities of dendritic cells. *Gastroenterology* (2011) **141**(1):176–85. doi:10.1053/j.gastro.2011.04.010
178. Zeng R, Oderup C, Yuan R, Lee M, Habtezion A, Hadeiba H, et al. Retinoic acid regulates the development of a gut-homing precursor for intestinal dendritic cells. *Mucosal Immunol* (2013) **6**(4):847–56. doi:10.1038/mi.2012.123
179. Villablanca EJ, De Calisto J, Torregrosa Paredes P, Cassani B, Nguyen DD, Gabrielsson S, et al. Beta7 integrins are required to give rise to intestinal mononuclear phagocytes with tolerogenic potential. *Gut* (2014) **63**(9):1431–40. doi:10.1136/gutjnl-2013-305386
180. Raman M, Milestone AN, Walters JR, Hart AL, Ghosh S. Vitamin D and gastrointestinal diseases: inflammatory bowel disease and colorectal cancer. *Therap Adv Gastroenterol* (2011) **4**(1):49–62. doi:10.1177/1756283X10377820
181. Demay MB. Mechanism of vitamin D receptor action. *Ann N Y Acad Sci* (2006) **1068**:204–13. doi:10.1196/annals.1346.026
182. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. *J Immunol* (2007) **179**(3):1634–47. doi:10.4049/jimmunol.179.3.1634
183. Bhalla AK, Amento EP, Serog B, Glimcher LH. 1,25-dihydroxyvitamin D3 inhibits antigen-induced T cell activation. *J Immunol* (1984) **133**(4):1748–54.
184. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1alpha,25-dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J Immunol* (2001) **167**(9):4974–80. doi:10.4049/jimmunol.167.9.4974
185. Daniel C, Sartory NA, Zahn N, Radeke HH, Stein JM. Immune modulatory treatment of trinitrobenzene sulfonic acid colitis with calcitriol is associated with a change of a T helper (Th) 1/Th17 to a Th2 and regulatory T cell profile. *J Pharmacol Exp Ther* (2008) **324**(1):23–33. doi:10.1124/jpet.107.127209
186. Penna G, Roncari A, Amuchastegui S, Daniel KC, Berti E, Colonna M, et al. Expression of the inhibitory receptor ILT3 on dendritic cells is dispensable for induction of CD4+Foxp3+ regulatory T cells by 1,25-dihydroxyvitamin D3. *Blood* (2005) **106**(10):3490–7. doi:10.1182/blood-2005-05-2044
187. Almerighi C, Sinistro A, Cavazza A, Ciaprini C, Rocchi G, Bergamini A. 1alpha,25-dihydroxyvitamin D3 inhibits CD40L-induced pro-inflammatory and immunomodulatory activity in human monocytes. *Cytokine* (2009) **45**(3):190–7. doi:10.1016/j.cyto.2008.12.009
188. Szeles L, Keresztes G, Torocsik D, Balajthy Z, Krenacs L, Poliska S, et al. 1,25-dihydroxyvitamin D3 is an autonomous regulator of the transcriptional changes leading to a tolerogenic dendritic cell phenotype. *J Immunol* (2009) **182**(4):2074–83. doi:10.4049/jimmunol.0803345
189. Ananthakrishnan AN. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol Hepatol* (2015) **12**(4):205–17. doi:10.1038/nrgastro.2015.34
190. Sentongo TA, Semaeo EJ, Stettler N, Piccoli DA, Stallings VA, Zemel BS. Vitamin D status in children, adolescents, and young adults with Crohn disease. *Am J Clin Nutr* (2002) **76**(5):1077–81.
191. Simmons JD, Mullighan C, Welsh KI, Jewell DP. Vitamin D receptor gene polymorphism: association with Crohn's disease susceptibility. *Gut* (2000) **47**(2):211–4. doi:10.1136/gut.47.2.211
192. Sigmundsdottir H, Pan J, Debes GF, Alt C, Habtezion A, Soler D, et al. DCs metabolize sunlight-induced vitamin D3 to 'program' T cell attraction to the epidermal chemokine CCL27. *Nat Immunol* (2007) **8**(3):285–93. doi:10.1038/ni1433
193. Froicu M, Zhu Y, Cantorna MT. Vitamin D receptor is required to control gastrointestinal immunity in IL-10 knockout mice. *Immunology* (2006) **117**(3):310–8. doi:10.1111/j.1365-2567.2005.02290.x
194. Kong J, Zhang Z, Musch MW, Ning G, Sun J, Hart J, et al. Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. *Am J Physiol Gastrointest Liver Physiol* (2008) **294**(1):G208–16. doi:10.1152/ajpgi.00398.2007
195. Wu S, Zhang YG, Lu R, Xia Y, Zhou D, Petrof EO, et al. Intestinal epithelial vitamin D receptor deletion leads to defective autophagy in colitis. *Gut* (2014) **64**(7):1082–94. doi:10.1136/gutjnl-2014-307436
196. Ananthakrishnan AN, Khalili H, Higuchi LM, Bao Y, Korzenik JR, Giovannucci EL, et al. Higher predicted vitamin D status is associated with reduced risk of Crohn's disease. *Gastroenterology* (2012) **142**(3):482–9. doi:10.1053/j.gastro.2011.11.040

197. Frericks M, Meissner M, Esser C. Microarray analysis of the AHR system: tissue-specific flexibility in signal and target genes. *Toxicol Appl Pharmacol* (2007) **220**(3):320–32. doi:10.1016/j.taap.2007.01.014
198. Stockinger B, Di Meglio P, Gialitakis M, Duarte JH. The aryl hydrocarbon receptor: multitasking in the immune system. *Annu Rev Immunol* (2014) **32**:403–32. doi:10.1146/annurev-immunol-032713-120245
199. Moura-Alves P, Fae K, Houthuys E, Dorhoi A, Kreuchwig A, Furkert J, et al. AhR sensing of bacterial pigments regulates antibacterial defence. *Nature* (2014) **512**(7515):387–92. doi:10.1038/nature13684
200. Li Y, Innocentin S, Withers DR, Roberts NA, Gallagher AR, Grigorieva EF, et al. Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation. *Cell* (2011) **147**(3):629–40. doi:10.1016/j.cell.2011.09.025
201. Quintana FJ, Basso AS, Iglesias AH, Korn T, Farez MF, Bettelli E, et al. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* (2008) **453**(7191):65–71. doi:10.1038/nature06880
202. Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* (2008) **453**(7191):106–9. doi:10.1038/nature06881
203. Gandhi R, Kumar D, Burns EJ, Nadeau M, Dake B, Laroni A, et al. Activation of the aryl hydrocarbon receptor induces human type 1 regulatory T cell-like and Foxp3(+) regulatory T cells. *Nat Immunol* (2010) **11**(9):846–53. doi:10.1038/ni.1915
204. Qiu J, Heller JJ, Guo X, Chen ZM, Fish K, Fu YX, et al. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity* (2012) **36**(1):92–104. doi:10.1016/j.immuni.2011.11.011
205. Qiu J, Guo X, Chen ZM, He L, Sonnenberg GF, Artis D, et al. Group 3 innate lymphoid cells inhibit T-cell-mediated intestinal inflammation through aryl hydrocarbon receptor signaling and regulation of microflora. *Immunity* (2013) **39**(2):386–99. doi:10.1016/j.immuni.2013.08.002
206. Kiss EA, Vonarbourg C, Kopfmann S, Hobeika E, Finke D, Esser C, et al. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. *Science* (2011) **334**(6062):1561–5. doi:10.1126/science.1214914
207. Klose CS, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, d'Hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORgammat+ innate lymphoid cells. *Nature* (2013) **494**(7436):261–5. doi:10.1038/nature11813
208. Kimura A, Naka T, Nakahama T, Chinen I, Masuda K, Nohara K, et al. Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. *J Exp Med* (2009) **206**(9):2027–35. doi:10.1084/jem.20090560
209. Huai W, Zhao R, Song H, Zhao J, Zhang L, Zhang L, et al. Aryl hydrocarbon receptor negatively regulates NLRP3 inflammasome activity by inhibiting NLRP3 transcription. *Nat Commun* (2014) **5**:4738. doi:10.1038/ncomms5738
210. Wu C, Yosef N, Thalhamer T, Zhu C, Xiao S, Kishi Y, et al. Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1. *Nature* (2013) **496**(7446):513–7. doi:10.1038/nature11984
211. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci Transl Med* (2009) **1**(6):6ra14. doi:10.1126/scitranslmed.3000322
212. Bhowmik SK, An JH, Lee SH, Jung BH. Alteration of bile acid metabolism in pseudo germ-free rats [corrected]. *Arch Pharm Res* (2012) **35**(11):1969–77. doi:10.1007/s12272-012-1114-7
213. Brestoff JR, Artis D. Commensal bacteria at the interface of host metabolism and the immune system. *Nat Immunol* (2013) **14**(7):676–84. doi:10.1038/ni.2640
214. Belcheva A, Irrazabal T, Robertson SJ, Streutker C, Maughan H, Rubino S, et al. Gut microbial metabolism drives transformation of MSH2-deficient colon epithelial cells. *Cell* (2014) **158**(2):288–99. doi:10.1016/j.cell.2014.04.051
215. Schulz MD, Atay C, Heringer J, Romrig FK, Schwitalla S, Aydin B, et al. High-fat-diet-mediated dysbiosis promotes intestinal carcinogenesis independently of obesity. *Nature* (2014) **514**(7523):508–12. doi:10.1038/nature13398
216. Liu L, Li L, Min J, Wang J, Wu H, Zeng Y, et al. Butyrate interferes with the differentiation and function of human monocyte-derived dendritic cells. *Cell Immunol* (2012) **277**(1–2):66–73. doi:10.1016/j.cellimm.2012.05.011
217. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc Natl Acad Sci U S A* (2014) **111**(6):2247–52. doi:10.1073/pnas.1322269111
218. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, et al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* (2014) **40**(1):128–39. doi:10.1016/j.immuni.2013.12.007
219. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* (2013) **341**(6145):569–73. doi:10.1126/science.1241165
220. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* (2013) **504**(7480):451–5. doi:10.1038/nature12726
221. Barbi J, Pardoll D, Pan F. Metabolic control of the Treg/Th17 axis. *Immunol Rev* (2013) **252**(1):52–77. doi:10.1111/imr.12029
222. Berod L, Friedrich C, Nandan A, Freitag J, Hagemann S, Harmrolfs K, et al. De novo fatty acid synthesis controls the fate between regulatory T and T helper 17 cells. *Nat Med* (2014) **20**(11):1327–33. doi:10.1038/nm.3704
223. Berndt BE, Zhang M, Owyang SY, Cole TS, Wang TW, Luther J, et al. Butyrate increases IL-23 production by stimulated dendritic cells. *Am J Physiol Gastrointest Liver Physiol* (2012) **303**(12):G1384–92. doi:10.1152/ajpgi.00540.2011
224. Huda-Faujan N, Abdulamir AS, Fatimah AB, Anas OM, Shuhaimi M, Yazid AM, et al. The impact of the level of the intestinal short chain fatty acids in inflammatory bowel disease patients versus healthy subjects. *Open Biochem J* (2010) **4**:53–8. doi:10.2174/1874091X01004010053
225. Sokol H, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, et al. Low counts of *Faecalibacterium prausnitzii* in colitis microbiota. *Inflamm Bowel Dis* (2009) **15**(8):1183–9. doi:10.1002/ibd.20903
226. Hamer HM, Jonkers DM, Vanhoutvin SA, Troost FJ, Rijkers G, de Bruine A, et al. Effect of butyrate enemas on inflammation and antioxidant status in the colonic mucosa of patients with ulcerative colitis in remission. *Clin Nutr* (2010) **29**(6):738–44. doi:10.1016/j.clnu.2010.04.002
227. Scheppach W, Sommer H, Kirchner T, Paganelli GM, Bartram P, Christl S, et al. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. *Gastroenterology* (1992) **103**(1):51–6.
228. Vinolo MA, Rodrigues HG, Nachbar RT, Curi R. Regulation of inflammation by short chain fatty acids. *Nutrients* (2011) **3**(10):858–76. doi:10.3390/nu3100858
229. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med* (2014) **20**(2):159–66. doi:10.1038/nm.3444
230. Tall AR, Yvan-Charvet L. Cholesterol, inflammation and innate immunity. *Nat Rev Immunol* (2015) **15**(2):104–16. doi:10.1038/nri3793
231. Vavassori P, Mencarelli A, Renga B, Distrutti E, Fiorucci S. The bile acid receptor FXR is a modulator of intestinal innate immunity. *J Immunol* (2009) **183**(10):6251–61. doi:10.4049/jimmunol.0803978
232. Gadaleta RM, van Erpecum KJ, Oldenburg B, Willemsen EC, Renooij W, Murzilli S, et al. Farnesoid X receptor activation inhibits inflammation and preserves the intestinal barrier in inflammatory bowel disease. *Gut* (2011) **60**(4):463–72. doi:10.1136/gut.2010.212159
233. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat Med* (2003) **9**(2):213–9. doi:10.1038/nm820
234. A-Gonzalez N, Bensinger SJ, Hong C, Beceiro S, Bradley MN, Zelcer N, et al. Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. *Immunity* (2009) **31**(2):245–58. doi:10.1016/j.immuni.2009.06.018
235. Bensinger SJ, Bradley MN, Joseph SB, Zelcer N, Janssen EM, Hausner MA, et al. LXR signaling couples sterol metabolism to proliferation in the acquired immune response. *Cell* (2008) **134**(1):97–111. doi:10.1016/j.cell.2008.04.052
236. Soroosh P, Wu J, Xue X, Song J, Sutton SW, Sablad M, et al. Oxysterols are agonist ligands of RORgammat and drive Th17 cell differentiation. *Proc Natl Acad Sci U S A* (2014) **111**(33):12163–8. doi:10.1073/pnas.1322807111
237. Weiner HL, da Cunha AP, Quintana F, Wu H. Oral tolerance. *Immunol Rev* (2011) **241**(1):241–59. doi:10.1111/j.1600-065X.2011.01017.x

238. Faria AM, Weiner HL. Oral tolerance: therapeutic implications for autoimmune diseases. *Clin Dev Immunol* (2006) **13**(2–4):143–57. doi:10.1080/17402520600876804
239. Mazzini E, Massimiliano L, Penna G, Rescigno M. Oral tolerance can be established via gap junction transfer of fed antigens from CX3CR1(+) macrophages to CD103(+) dendritic cells. *Immunity* (2014) **40**(2):248–61. doi:10.1016/j.immuni.2013.12.012
240. Shan M, Gentile M, Yeiser JR, Walland AC, Bornstein VU, Chen K, et al. Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. *Science* (2013) **342**(6157):447–53. doi:10.1126/science.1237910
241. Worbs T, Bode U, Yan S, Hoffmann MW, Hintzen G, Bernhardt G, et al. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *J Exp Med* (2006) **203**(3):519–27. doi:10.1084/jem.20052016
242. Manicassamy S, Reizis B, Ravindran R, Nakaya H, Salazar-Gonzalez RM, Wang YC, et al. Activation of beta-catenin in dendritic cells regulates immunity versus tolerance in the intestine. *Science* (2010) **329**(5993):849–53. doi:10.1126/science.1188510
243. Mortha A, Chudnovskiy A, Hashimoto D, Bogunovic M, Spencer SP, Belkaid Y, et al. Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. *Science* (2014) **343**(6178):1249288. doi:10.1126/science.1249288
244. Stefka AT, Feehley T, Tripathi P, Qiu J, McCoy K, Mazmanian SK, et al. Commensal bacteria protect against food allergen sensitization. *Proc Natl Acad Sci U S A* (2014) **111**(36):13145–50. doi:10.1073/pnas.1412008111
245. Esplugues E, Huber S, Gagliani N, Hauser AE, Town T, Wan YY, et al. Control of TH17 cells occurs in the small intestine. *Nature* (2011) **475**(7357):514–8. doi:10.1038/nature10228
246. Wang J, Li F, Wei H, Lian ZX, Sun R, Tian Z. Respiratory influenza virus infection induces intestinal immune injury via microbiota-mediated Th17 cell-dependent inflammation. *J Exp Med* (2014) **211**(12):2397–410. doi:10.1084/jem.20140625
247. Noval Rivas M, Burton OT, Wise P, Charbonnier LM, Georgiev P, Oettgen HC, et al. Regulatory T cell reprogramming toward a Th2-cell-like lineage impairs oral tolerance and promotes food allergy. *Immunity* (2015) **42**(3):512–23. doi:10.1016/j.immuni.2015.02.004
248. da Cunha AP, Wu HY, Rezende RM, Vandeventer T, Weiner HL. In vivo anti-LAP mAb enhances IL-17/IFN-gamma responses and abrogates anti-CD3-induced oral tolerance. *Int Immunol* (2015) **27**(2):73–82. doi:10.1093/intimm/dxu083
249. Benson JM, Campbell KA, Guan Z, Gienapp IE, Stuckman SS, Forsthuber T, et al. T-cell activation and receptor downmodulation precede deletion induced by mucosally administered antigen. *J Clin Invest* (2000) **106**(8):1031–8. doi:10.1172/JCI10738
250. Macian F, Garcia-Cozar F, Im SH, Horton HF, Byrne MC, Rao A. Transcriptional mechanisms underlying lymphocyte tolerance. *Cell* (2002) **109**(6):719–31. doi:10.1016/S0092-8674(02)00767-5
251. Kriegel MA, Rathinam C, Flavell RA. E3 ubiquitin ligase GRAIL controls primary T cell activation and oral tolerance. *Proc Natl Acad Sci U S A* (2009) **106**(39):16770–5. doi:10.1073/pnas.0908957106
252. Dubois B, Joubert G, Gomez de Aguero M, Gouanvic M, Goubier A, Kaiserlian D. Sequential role of plasmacytoid dendritic cells and regulatory T cells in oral tolerance. *Gastroenterology* (2009) **137**(3):1019–28. doi:10.1053/j.gastro.2009.03.055

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