INDUCING IMMUNE TOLERANCE TO THERAPEUTIC PROTEINS, CELLS AND TISSUES

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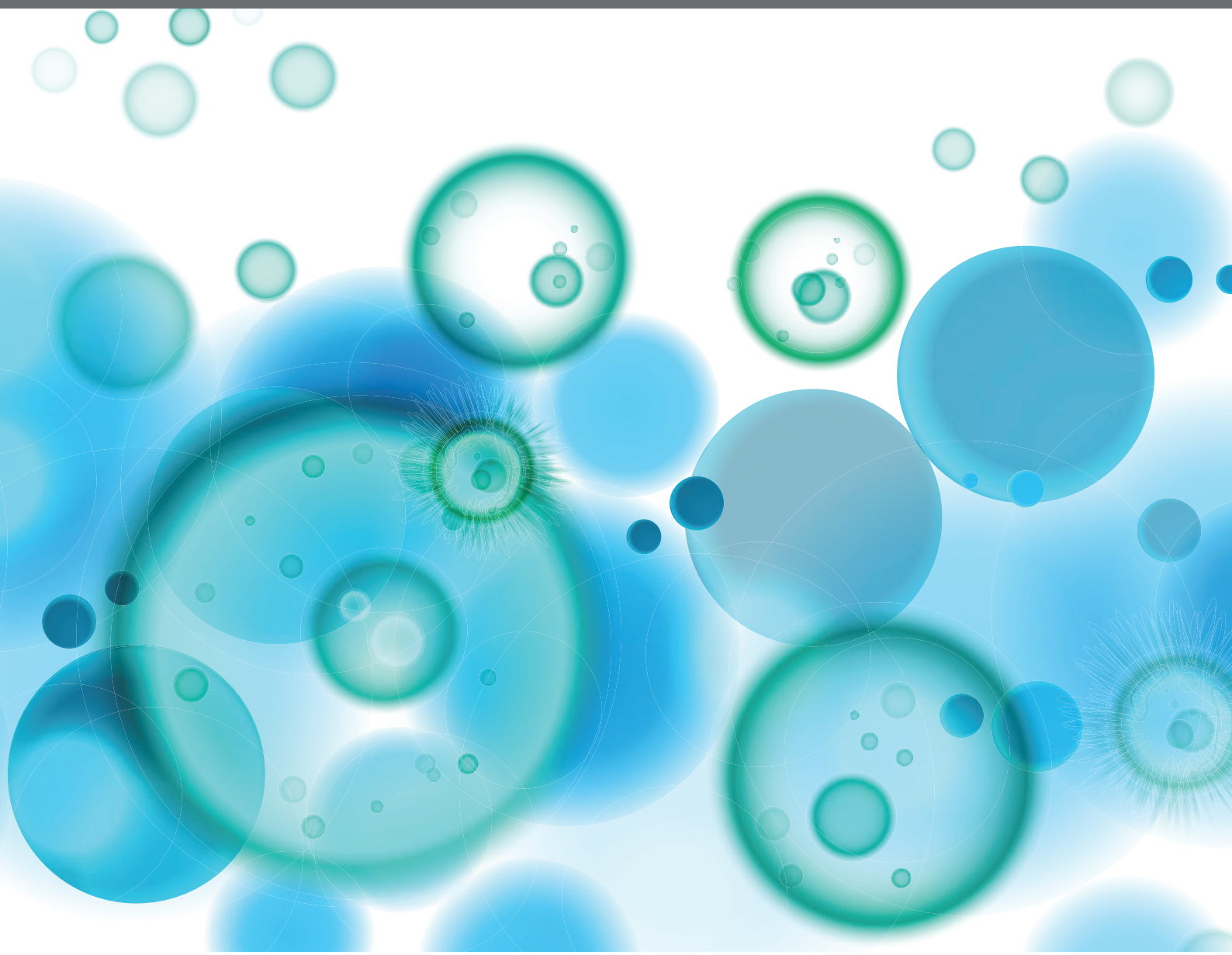

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INDUCING IMMUNE TOLERANCE TO THERAPEUTIC PROTEINS, CELLS AND TISSUES

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Escherichia coli Heat-Labile Enterotoxin B Limits T Cells Activation by Promoting Immature Dendritic Cells and Enhancing Regulatory T Cell Function

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Treatments to limit T cell activation are essential for managing autoimmune and inflammatory disorders. The B subunit of *Escherichia coli* heat-labile enterotoxin (EtxB) is known to ameliorate inflammatory disease *in vivo* but the mechanism by which this is mediated is not well understood. Here, we show that following intranasal administration, EtxB acts on two key cellular regulators of T cell activation: regulatory T cells and dendritic cells (DCs). EtxB enhances the proliferation of lung regulatory T cells and doubles their suppressive function, likely through an increase in expression of the Treg effector molecule CTLA-4. EtxB supports the generation of interleukin-10-producing DCs that are unable to activate T cells. These data show, for the first time, that mucosal EtxB treatment limits T cells activation by acting jointly on two distinct types of immune cells.

Keywords: EtxB, immunomodulation, lung, dendritic cells, regulatory T cells

INTRODUCTION

Robust immunological tolerance is essential to prevent development of autoimmune and autoinflammatory disorders. The challenge of the immune system is to balance potent effector mechanisms against foreign pathogens, while remaining unresponsive to self-antigens. The mucosal immune system faces a more complex challenge in that it must also regulate commensal microbial communities that are essential for health, in addition to discriminating between self- and non-self-antigens (1, 2). This balance is maintained by a number of cell types, including Foxp3$^+$ regulatory T (Treg) cells and immature or tolerogenic dendritic cells (DCs) (3, 4). Treg cells are a population of suppressive CD4$^+$ T cells that act to limit the effector functions of other leukocytes such as CD4$^+$ T cells, thereby limiting inflammation and tissue damage. Within the mucosa, Treg cells modulate inflammatory responses by producing high levels of interleukin-10 (IL-10), restraining the generation of inflammatory disease (5–7). Tolerogenic DCs are antigen-presenting cells that are thought to able to promote T cell tolerance to a defined antigen. Typically, tolerogenic DCs have an "immature" phenotype, expressing lower levels of the cell surface receptors that are required to activate T cells (8). In the lung, DCs remain in an immature state and fail to activate naïve T cells, unless they receive an activating signal in parallel with antigen (9). However, whether a specific tolerogenic DC lineage exists *in vivo* or whether this is simply a modification of their activation status is unclear; because of this we will refer to these cells as immature DCs (10). Previous studies show that immature DCs may regulate immunological tolerance through different mechanisms such as the induction of T cell

anergy, generation of Treg cells and *via* production of IL-10 and transforming growth factor (TGF)-β (4, 11–13). In addition, immature DCs can support Treg cell differentiation *in vivo* through presentation of low levels of antigen in major histocompatibility complex (MHC)-II (14–16). Therapeutic strategies that augment numbers and/or function of Treg cells, immature DCs, or both, represent a way to enhance mucosal tolerance by limiting T cell activation.

The *Escherichia coli* heat-labile enterotoxin is a hetero-oligomeric AB_5 toxin composed of a toxic enzymatic A subunit and five identical non-toxic B subunits (EtxB) (17). In the context of infection, the B subunit mediates cellular entry of the A subunit into the cytoplasm by binding to GM1 ganglioside receptor, which is ubiquitously expressed by all somatic cells (18). Several studies have demonstrated the immunomodulatory effects of EtxB, with emphasis on its adjuvant properties, although the mechanism by which EtxB act as an adjuvant is not yet known (19–22). Previous studies reported that recombinant EtxB is non-toxic and its effects are dependant on EtxB binding to cell surface receptors, as evidenced by a failure of a non-receptor-binding mutant, EtxB (G33D), to induce any immunomodulatory effects (23–27). At the cellular level, one study suggests that EtxB binding to GM1 receptor induces both caspase-dependent and -independent cell death pathway in $CD8^+$ T cells (28). Conversely, a different study highlighted that receptor occupancy by EtxB on B cells is associated with maintenance of B-cell survival by activation of molecules essential for B-cell differentiation (29). Interestingly, binding of EtxB to GM1 receptor seems to be essential for EtxB-mediated antigen presentation by a immortalized murine bone marrow-derived dendritic cell (BMDC) line; however, EtxB did not induce maturation of BMDC (30, 31). At the molecular level, one study reported that receptor binding by EtxB triggers MAPK/ERK kinase activation in B cells (27). However, the precise molecular mechanisms by which EtxB induces direct or indirect effects on immune cells are largely unknown, in particular on DCs and Treg cells. Nevertheless, mucosal administration of EtxB ameliorates the disease severity of type 1 diabetes and collagen-induced arthritis in mice (23, 25). Treatment of these autoimmune mouse models with EtxB administration has been recapitulated by the transfer of splenocytes from EtxB-treated mice. Interestingly, when these splenocytes were devoid of $CD4^+$ T cells they could not mediate tolerance, suggesting a role for EtxB in modulating suppressive Treg cells. In support of this model, intranasal (i.n.) administration of EtxB increased the frequency of $Foxp3^+$ cells within the $CD4^+$ T cell population (24, 26). Together, these studies suggest that EtxB supports tolerance through increasing Treg cell number. However, the mechanism by which EtxB does this has not been determined, nor is it known if EtxB can alter the suppressive capacity of Treg cells.

In addition, i.n. administration of EtxB induces IL-10 and TGF-β1 production by both epithelial cells in nasal-associated lymphoid tissue and $CD11b^+$ cells in the cervical lymph nodes which suggest that EtxB may promote a tolerogenic environment (26). *In vitro* EtxB treatment increases viability of DCs and results in lower expression of MHC class II, CD80, and CD86 features of an immature phenotype (32). This suggests that in addition to enhancing Treg cell proportion, EtxB may also promote immature DCs *in vivo*, although this has not yet been demonstrated.

Interestingly, oral, nasal, or sublingual coadministration of the related non-toxic B subunit protein of cholera toxin (CTB) with selected antigens have been found to induce tolerance, including in autoimmune disorders and allergies in several animal models (33). One of the suggested mechanisms is that the coadministration of CTB with antigen results in increase of antigen-specific $Foxp3^+$ Treg cells (33). In addition, CTB diminishes the responsiveness of macrophages and monocytes to lipopolysaccharide (LPS) (34).

Our study aimed to determine the mechanism by which EtxB treatment promotes the accumulation of Treg cells at mucosal sites and whether EtxB treatment affects Treg cell function and DC activation status and function. We confirm that i.n. EtxB treatment increases the proportion of IL-10^+ $Foxp3^+$ Treg cells and, for the first time, show that this treatment increased the suppressive function of Treg cells, likely through increases in expression of the key Treg effector molecule CTLA-4 (cytotoxic T-lymphocyte-associated protein 4). In addition, mucosal administration of EtxB also increases the frequency of $CD8^-$ cDCs with an immature phenotype and enhances their ability to produce IL-10. We show *in vitro* that EtxB directly promotes immature phenotype in BMDCs that fail to activate naïve $CD4^+$ T cells. Together, these data demonstrate that EtxB alters the cellular composition of the lung, promoting a regulatory environment that is likely the cause of the anti-inflammatory activity of this protein.

MATERIALS AND METHODS

Experimental Animals

C57BL/6, TCR7 (35), and ITIB mice (36) (provided by H. Bouabe and K. Okkenhaug) were housed under specific pathogen-free conditions at the Biological Support Unit, Babraham Research Campus, Cambridge, UK. All experiments were approved by the UK Home Office under the UK Home Office license PPL 80/2526, in line with the Scientific Procedures Act (1986).

Administration of EtxB

EtxB-endotoxin free (Trident Pharmaceuticals, USA) was administered by the intranasal route under inhaled isoflurane anesthesia. Each mouse was administered 100 µg EtxB (as previously described), or heat-inactivated EtxB (95°C for 10 min), in 20 µl of sterile PBS or 20 µl of sterile PBS alone, on three consecutive days (23). The dose of 100 µg EtxB used i.n. has previously been shown to promote tolerance (23). Mice were euthanized at different time points after the last treatment, as indicated in the figure legend.

Flow Cytometry and Cell Sorting

Single-cell suspensions were prepared from mouse spleen by sieving and gentle pipetting through Falcon 70 µm nylon mesh filters (BD Biosciences, San Jose, CA, USA). To prepare cell suspensions from mediastinal lymph node (mLN), the tissue was incubated with 1 mg/ml Collagenase D (Roche Diagnostics, Mannheim, Germany), and 400 U/ml DNase I (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at room temperature, followed by

gentle pipetting to disrupt tissue. Lung lymphocytes were isolated by finely mincing the lung tissues and digesting with 2 mg/ml Collagenase (Sigma-Aldrich, St. Louis, MO, USA) and 0.2 mg/ml DNase I (Roche Diagnostics) at 37°C for 30 min, followed by sieving and gentle pipetting through Falcon 70 µm nylon mesh filters (BD Biosciences). Red blood cells were removed by using ammonium chloride lysis buffer. Cells were washed with PBS with 2% FCS, (PBS–FCS 2%) then stained with antibody cocktails. Different gating strategies were used to define myeloid and common DC subsets as previously described (32) (Figure S2 in Supplementary Material). For intracellular staining, cell suspensions were fixed and permeabilized using the Intracellular Fixation and Permeabilization Buffer Set as per the manufacturer's instructions (eBioscience, San Diego, CA, USA). Annexin V and DAPI staining procedure was performed following *Annexin V Staining Protocol* from BD Biosciences. To assess IL-10 production *ex vivo*, we used IL-10-β-lactamase reporter mice (ITIB) (36) as described previously. Briefly, IL-10 was detected in cells from ITIB mice using CCF4-AM staining solution supplemented with probenecid, prepared according to the manufacturer's instructions (Thermo Scientific, Wilmington, DE, USA), and incubated for 75 min at room temperature. Cells were washed with PBS–FCS 2% and analyzed by flow cytometry. For cell sorting, conventional naïve $CD4^+$ T cells ($B220^-CD3^+CD4^+CD25^-CD62L^+$) or regulatory T cells ($B220^-CD3^+CD4^+CD25^+$) were sorted from the spleen or the lung, respectively, after cell surface staining. FACS was performed with a FACSAria cell sorter (BD Biosciences) and sorted populations were between 95 and 99% purity. Analyses were carried out on an LSR Fortessa (BD Biosciences) using FlowJo software (TreeStar, Ashand, OR, USA).

Antibodies and Dyes for Flow Cytometry

Antibodies for flow cytometry were from eBioscience except where otherwise indicated: anti-CD4 (Biolegend, London, UK, RM4-5), anti-CD8 (Becton Dickinson, 53-6.7), anti-Foxp3 (FJK-16S), anti-Ki-67 (SolA15), anti-CTLA-4 (UC10-4B01), anti-CD304 (3DS304M), anti-CD25 (PC61.5), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-CD11b (M1/70), anti-CD11c (N418), anti-MHC-II (M5/114.15.2), anti-CD69 (H1.2F3), Annexin V, and DAPI.

Regulatory T Cell Suppression Assay

Regulatory T cell suppression assay was performed as previously published (37). Briefly, conventional splenic $CD4^+$ T cells were isolated from untreated C57BL/6 mice and labeled with 10 µM CellTrace Violet (eBioscience). Conventional $CD4^+$ T cells (2.5×10^4/well) were activated with anti-CD3 anti-CD28 coated beads (Gibco by Life Technologies, AR, Oslo) and cultured either alone or with lung $CD4^+CD25^+$ regulatory T cells at a range of ratios from 1:1 to 64:1 ($CD4^+CD25^-$: $CD4^+CD25^+$). After 4 days of culture, proliferation of conventional $CD4^+$ T cells was determined by CellTrace violet dilution by flow cytometry and the percentage of suppression of $CD4^+CD25^+$ T cells can be calculated using the following formula: [(% of proliferation of $CD4^+CD25^-$ cells alone − % of proliferation of $CD4^+CD25^-$ treated with $CD4^+CD25^+$)/% of proliferation of $CD4^+CD25^-$ cells alone] as previously described (37).

BMDC Culture and Treatment

Bone marrow cells were obtained from the femur and tibia of untreated C57BL/6 mice (38). To generate BMDCs, the cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 20 ng/ml of GM-CSF (R&D systems, Abingdon, UK), 10 ng/ml of IL-4 (R&D systems) and 50 µM of 2-Mercaptoethanol (Sigma-Aldrich) for 6 days. To induce activation, BMDCs were treated with 1 mg/ml of LPS (Sigma-Aldrich) for three additional days of culture. To generate tolerogenic DC, bone marrow cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 20 ng/ml of GM-CSF, 20 ng/ml of IL-10, 20 ng/ml of TGF-β (R&D systems), and 50 µM of 2-Mercaptoethanol for 9 days. BMDC cells were cultured for 12 h with hEtxB or EtxB (both at 10 µg/ml) at 37°C in 5% CO_2. The concentration of hEtxB or EtxB used *in vitro* and the time of treatment was consistent with previously published studies (32).

Antigen-Specific Presentation Assay

Hen egg lysozyme (HEL) or ovalbumin (OVA) (Sigma-Aldrich) were added to BMDCs at 1 mg/ml for 24 h, cells were fixed in 0.75% paraformaldehyde for 30 min on ice. Naïve TCR7 $CD4^+$ T cells were isolated using a MagniSort™ Mouse CD4 Naïve T cell Enrichment Kit (eBioscience) according to the manufacturer's instructions. 9×10^4 BMDCs that were exposed to HEL or OVA were co-cultured with 2.5×10^5 naïve $CD4^+$ transgenic T cells in RPMI-1640 containing 5% FCS (Fixed BMDCs:$CD4^+$ T cells = 1:3). $CD4^+$ T cell activation was assessed by flow cytometry at 5 h post co-culture by membrane expression of CD69.

Statistics

Data are presented as mean ± SD. Single comparisons were analyzed using the non-parametric Mann–Whitney *U*-test. All statistical analyses were carried out with GraphPad Prism v6 (La Jolla, CA, USA).

RESULTS

The Proportion of $Foxp3^+$ Regulatory T Cells Increase Following Intranasal EtxB Treatment

$Foxp3^+$ Treg cells are powerful mediators of immunological tolerance; their frequency has been reported to increase upon EtxB treatment (24, 26). We first sought to confirm this following i.n. EtxB administration. In the lung, EtxB treatment results in an increased proportion of $CD4^+$ T cells expressing Foxp3 2.5 days after treatment (**Figures 1A,B**) compared to control mice that received PBS or biologically inactive heat-treated EtxB (hEtxB). By contrast, the increased frequency of Treg cells is not detectable 2.5 days posttreatment in the draining mLN or spleen (**Figure 1B**). Interestingly, an increased proportion of Tregs in the spleen is detectable at day 9 posttreatment (Figure S1A in Supplementary Material), suggesting that the mucosal site of administration is the first and major location of EtxB action on Treg cells. Interestingly, no differences in the spleen, the mLN, or in the lung were observed between PBS- and hEtxB-treated mice at day 2.5 following treatment indicating that hEtxB is a relevant

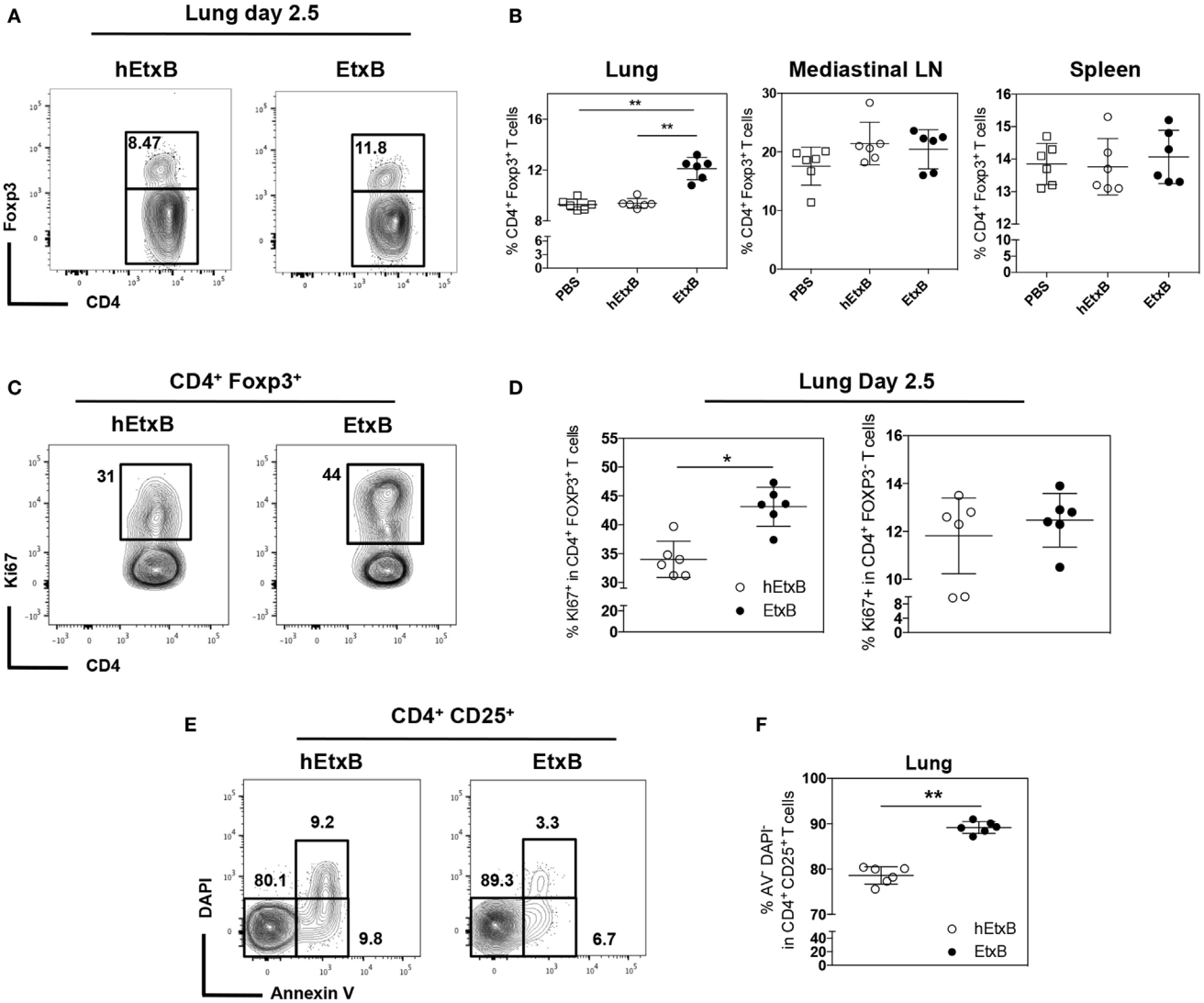

FIGURE 1 | Intranasal treatment of EtxB increases the proliferation of Treg cells. C57BL/6 mice were administered, EtxB (100 μg/mouse), heat inactivated EtxB (hEtxB, 100 μg/mouse) or PBS on three consecutive days. On day 2.5 following the last dose, spleen, mediastinal lymph node, and lung were removed and analyzed by flow cytometry. Flow cytometric contour plots **(A)** and dot plots **(B)** show the frequency of Foxp3$^+$ regulatory T (Treg) cells in CD4$^+$ B220$^-$ cells. The frequency of Ki67$^+$ proliferating Treg cells or CD4$^+$ Foxp3$^-$ T cells **(C,D)** and frequency of live (Annexin V$^-$ DAPI$^-$) CD4$^+$ CD25$^+$ cells **(E,F)** in the lung. Results are from one experiment representative of three independent experiments, with six mice per group. In dot plots, the mean and SD are represented and each circle represents one mouse: white squares PBS treated animals, white dots hEtxB-treated animals, black dots EtxB-treated animals. *$p < 0.05$ and **$p < 0.005$ compared with PBS- or hEtxB-treated mice (Mann–Whitney *U*-test).

biologically inactive negative control (**Figure 1B**). While previous studies have reported an increase in Treg cell proportion following EtxB treatment, they have not determined how this occurs. This observation suggests that EtxB may be able to alter Treg cell proliferation and/or survival. To test this, we determined the frequency of proliferating (Ki67$^+$) and live (Annexin V$^-$DAPI$^-$) Treg cells in the lung 2.5 days after EtxB administration. After EtxB treatment, Treg cells had a significantly increased proportion of both Ki67$^+$ cells (**Figures 1C,D**) and live cells (**Figures 1E,F**). By contrast, EtxB administration is not able to induce the proliferation of CD4$^+$ Foxp3$^-$ T cells suggesting an EtxB-Treg specific effect and that EtxB alone seems not to be "immunogenic" on naïve/effector CD4$^+$ T cells (**Figure 1D**). Together, the increase in cell proliferation with a decrease in cell death likely accounts for the higher proportion of Treg cells in the lung of EtxB-treated mice and that mucosal sites are the principle location of this effect.

The Suppressive Function of Treg Cells Is Enhanced by EtxB Treatment

Previous work has suggested that EtxB dampens inflammation through increased proportion of Treg cells (23–25), but it is also possible that EtxB functionally alters Treg cells. We sought to determine if mucosal administration of EtxB alters the activation

status and/or suppressive capacity of Treg cells. CTLA-4 is a key effector molecule for Treg cell function (39); Treg cells from EtxB-treated mice had increased expression of CTLA-4 compared to control hEtxB-treated mice in the lung, but the expression is unchanged in the mLN (**Figures 2A,B** and Figures S1B,C in Supplementary Material). In the lung, the expression of Neuropilin-1 on Treg cells is not affected in EtxB-treated mice suggesting no preferentially effect of EtxB on thymic Treg cells or induced peripheral regulatory T cells (**Figures 2C,D**). Several studies have shown that IL-10 plays a role in Treg cell suppressive function at mucosal sites (40, 41). To investigate the effect of i.n. EtxB administration on IL-10 production by Treg cells in the lung, we used an IL-10-β-lactamase (ITIB) reporter mouse (36). Treatment with EtxB resulted in a twofold increase in the frequency of IL-10-producing Treg cells, compared to control hEtxB-treated mice (**Figures 2E,F**). Interestingly, EtxB did not alter expression of other markers of Treg induction/activation such as inducible T-cell costimulator, programmed cell death 1, CD101, and CD103 (data not shown). Together, increased CTLA-4 and IL-10 expression suggests that EtxB is able to enhance the functional capacity of Treg cells. To test this hypothesis, we performed an *in vitro* Treg suppression assay (37). $CD4^+CD25^+$ Treg cells were isolated from the lung of EtxB-treated C57BL/6 mice by flow cytometric cell sorting and co-cultured with naïve $CD4^+$ T cells from an untreated animal, and the ability of Treg cells to suppress TCR-driven $CD4^+$ T cell proliferation was assessed. $CD4^+CD25^+$ Treg cells taken from EtxB-treated mice had twice the suppressive function of Treg cells isolated from hEtxB-treated control animals (**Figures 2G,H**). These findings demonstrate that mucosal administration of EtxB enhances the suppressive function of Treg cells, describing a novel role for EtxB in modifying Treg cell biology.

Mucosal EtxB Administration Increases Immature DCs

ExtB has previously been shown to modify innate cells, by reducing the frequency of DC precursors and myeloid precursors in secondary lymphoid tissues (32), and by increasing the expression of IL-10 and TGF-β transcripts in $CD11b^+$ cells (26). To confirm that EtxB acts on innate cells at mucosal sites, we examined the effect of i.n. EtxB administration on lung dendritic and myeloid cell subsets (Figure S2 in Supplementary Material). Consistent with the previous report (32), EtxB treatment reduced the proportion of conventional DC precursors (pre-cDC, $CD11c^{low}CD11b^+CD8^-MHC\text{-}II^-$) and myeloid cell precursors ($CD11b^+CD11c^-CD8^-MHC\text{-}II^-$) in the lung (Figures S3A,B in Supplementary Material). In addition, we also observed that expression of CD80 was upregulated in plasmacytoid pre-DCs (p-preDC, $CD11c^{low}CD11b^-CD8^-MHC\text{-}II^-$), pre-cDC, and myeloid precursors, while CD86 was elevated in pre-cDC in the lung of EtxB-treated mice (Figures S3C,D in Supplementary Material). In the mLN, EtxB treatment reduced only the proportion of myeloid cell precursors (Figures S4A,B in Supplementary Material) and had no effect on the expression of CD80 or CD86 in dendritic and myeloid cell precursors (Figures S4C,D in Supplementary Material). This suggests that EtxB can alter both the proportion and phenotype of innate immune cells in the lung and mLN.

Next, we investigated the effect of i.n. EtxB administration on mature DC subsets; plasmacytoid DC (pDC, $CD11c^{low}CD11b^-CD8^-MHC\text{-}II^+$), $CD8^-$, and $CD8^+$ cDCs ($CD11c^{high}CD11b^+CD8^{-/+}MHC\text{-}II^+$) in the lung and in the mLN. The proportions of these DC subsets were not affected by i.n. EtxB administration in either organ (**Figures 3A–C**). But, we observed that $CD8^-$cDC and pDC from EtxB-treated mice have significantly reduced expression of MHC class II, consistent with a profile of "immature" DCs (**Figures 4A–C**). The low levels of MHC class II on DCs from EtxB-treated mice is intriguing in the context of immunological tolerance, as DCs of this phenotype have also been described to have a poor capacity to activate T cells. Interestingly, the inability of immature DCs to activate T cells is partially dependent on IL-10 production by immature DCs (13, 16, 42). To investigate the ability of EtxB to promote IL-10 production by these immature phenotype DCs, we treated ITIB mice with EtxB or hEtxB as a control. EtxB-treated mice had a significantly increased percentage of $IL\text{-}10^+$ $CD8^-$cDC compared to hEtxB-treated control mice (**Figures 4D,E**). We did not observe any effect of EtxB treatment on IL-10 expression by the other subsets of DCs or myeloid cells (data not shown). These results show that mucosal administration of EtxB increases the proportion of "immature" $IL\text{-}10^+$ $CD8^-$ cDCs, a new immunoregulatory mechanism by which EtxB can modify the innate immune system at the mucosal surface.

EtxB Induces Immature BMDCs That Are Unable to Activate T Cells *In Vitro*

These data prompt the hypothesis that EtxB treatment results in an increase of immature DC population that fail to induce T cell activation, which may partly explain the regulatory effects of EtxB *in vivo*. To test this, we assessed the effect of EtxB on *in vitro* generated $CD11b^+$ $CD11c^+$ BMDCs. Bone marrow cells from C57BL/6 mice were cultured with GM-CSF and IL-4 (38) for 6 days prior to treatment with either RPMI alone or with hEtxB or EtxB followed by analysis of MHC class II expression (**Figure 5A**). As a positive control, we treated BMDCs with LPS to induce activation, leading to MHC-II upregulation. As a negative control, we generated BMDCs in tolerogenic culture conditions (with IL-10 and TGF-β), which reduced expression of MHC class II (38). EtxB treatment resulted in BMDCs that expressed a lower level of MHC class II compared to control cells. EtxB-treated BMDCs phenotypically resembled *in vitro*-induced immature BMDCs (**Figures 5B,C**). This suggests that EtxB can act directly on BMDCs to reduce expression of MHC class II and directly promotes an immature state thereby impeding their capacity to activate T cells. To test this, we performed an *in vitro* T cell activation assay with naive TCR7 transgenic $CD4^+$ T cells specific for the subdominant $H\text{-}2^b$ epitope of HEL. LPS-, IL-10-, hEtxB-, or EtxB-treated BMDCs were exposed to 1 mg/ml of HEL, or OVA antigen as negative control, for 24 h. The cells were washed, fixed, and added at a ratio 1:3 to TCR7 cells. Fixation was performed to ensure that the BMDCs did not mature during culture with T cells (43). Incubation of BMDCs

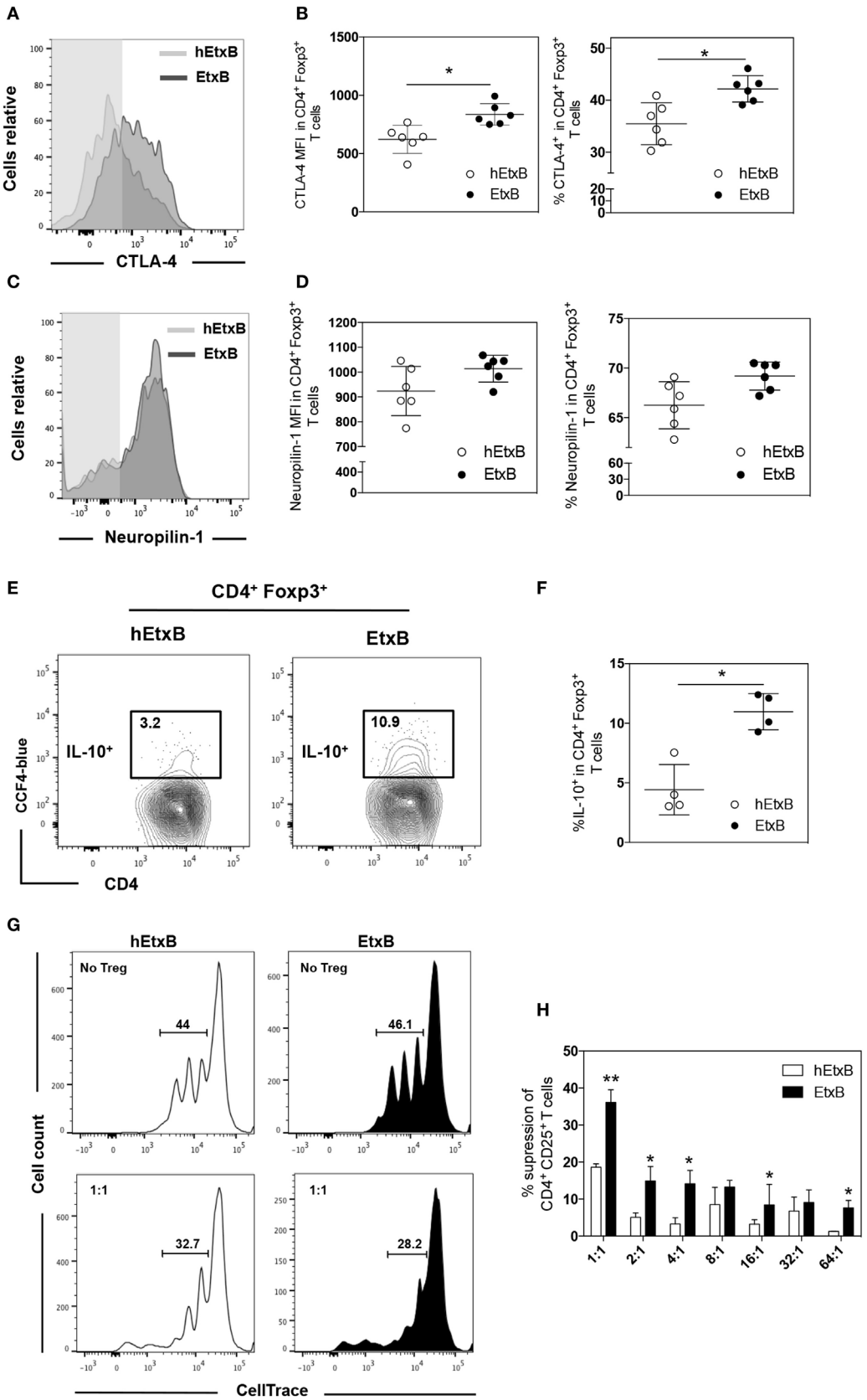

FIGURE 2 | **Continued**

FIGURE 2 | Continued

EtxB increases the suppressive function of Treg cells. Expression of CTLA-4 **(A,B)** or Neuropilin-1 **(C,D)** on lung B220$^-$ CD4$^+$ Foxp3$^+$ Treg cells from C57BL/6 mice treated intranasally with 100 μg/mouse EtxB or heat-EtxB (hEtxB) for three consecutive days, and analyzed by flow cytometry at 2.5 days after the final treatment. **(E,F)** Intranasal administration of 100 μg/mouse EtxB or hEtxB to ITIB mice. Day 2.5 posttreatment, lungs were removed and analyzed by flow cytometry for the proportion of IL-10$^+$ (CCF-4 blue) in B220$^-$ CD4$^+$ Foxp3$^+$ Treg cells. **(G,H)** Groups of 10 C57BL/6 mice were treated intranasally with 100 μg/mouse EtxB or hEtxB for three consecutive days. On day 2.5 posttreatment, lungs were removed and CD4$^+$ CD25$^+$ Treg cells were flow sorted and were co-cultured with splenic CD4$^+$ CD25$^-$ T cells isolated from untreated C57BL/6 mice that have been labeled with CellTrace Violet. Cells were cultured at the ratio of CD4$^+$CD25$^-$: CD4$^+$CD25$^+$ indicated on the graph. After 4 days, proliferation was determined by CellTrace Violet dilution **(E)** and the percentage of suppression of CD4$^+$ CD25$^+$ T cells was assessed using the following formula: [(% of proliferation of CD4$^+$CD25$^-$ cells alone − % of proliferation of CD4$^+$CD25$^-$ treated with CD4$^+$CD25$^+$)/% of proliferation of CD4$^+$CD25$^-$ cells alone] as previously described (37). Results are from one experiment representative of six **(A–D)** or four **(E,F)** mice in each group from three independent experiments. In histograms **(A,C)**, the shaded area represents the fluorescence minus one control. In dot plots, the mean and SD are represented and each circle represents one mouse: white dots hEtxB-treated animals, black dots EtxB-treated animals. Results in **(G,H)** are from one experiment representative of three independent experiments. *$p < 0.05$ and **$p < 0.005$ compared with hEtxB-treated mice (Mann–Whitney U-test).

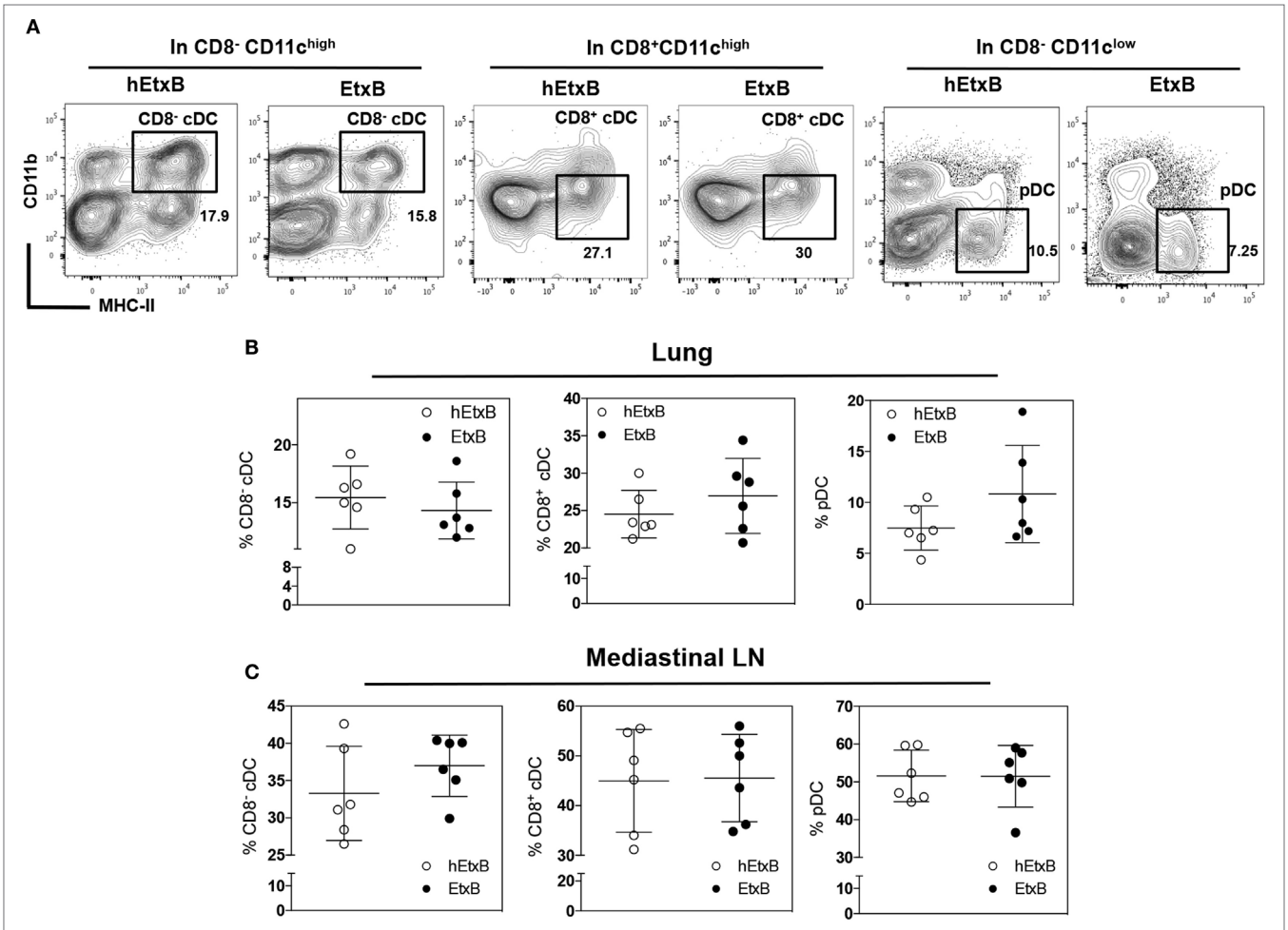

FIGURE 3 | EtxB does not alter mature dendritic cell (DC) subsets. C57BL/6 mice were treated intranasally with 100 μg/mouse EtxB or heat-EtxB (hEtxB) for 3 days. On day 2.5 posttreatment, lung and mediastinal lymph node (mLN) were removed and analyzed, flow cytometric contour plots **(A)** and dot plots show the proportion of DCs subsets in the lung **(B)** and mLN **(C)**. Subsets of DC include plasmacytoid DC (pDC, CD11c^{low}CD11b$^-$CD8$^-$MHC-II$^+$), CD8$^-$ and CD8$^+$ conventional DCs (CD8$^+$ or CD8$^-$ cDC, CD11c^{high}CD11b$^+$CD8$^{-/+}$ MHC-II$^+$). Results are from one experiment representative of six mice in each group from three independent experiments In dot plots, the mean and SD are represented and each circle represents one mouse: white dots hEtxB-treated animals, black dots EtxB-treated animals.

with HEL or OVA had no effect on MHC class II, CD80 and CD86 membrane expression (data not shown). As expected, in all conditions, BMDCs exposed to OVA antigen were unable to induce expression of the early activation marker, CD69, on TCR7 CD4$^+$ T cells (**Figures 5D,E**). BMDCs pulsed with HEL and activated with LPS were able to induce CD69 expression on T cells, while IL-10-induced BMDCs were not (**Figures 5D,E**). We found that HEL-pulsed hEtxB-BMDCs stimulated CD69 expression

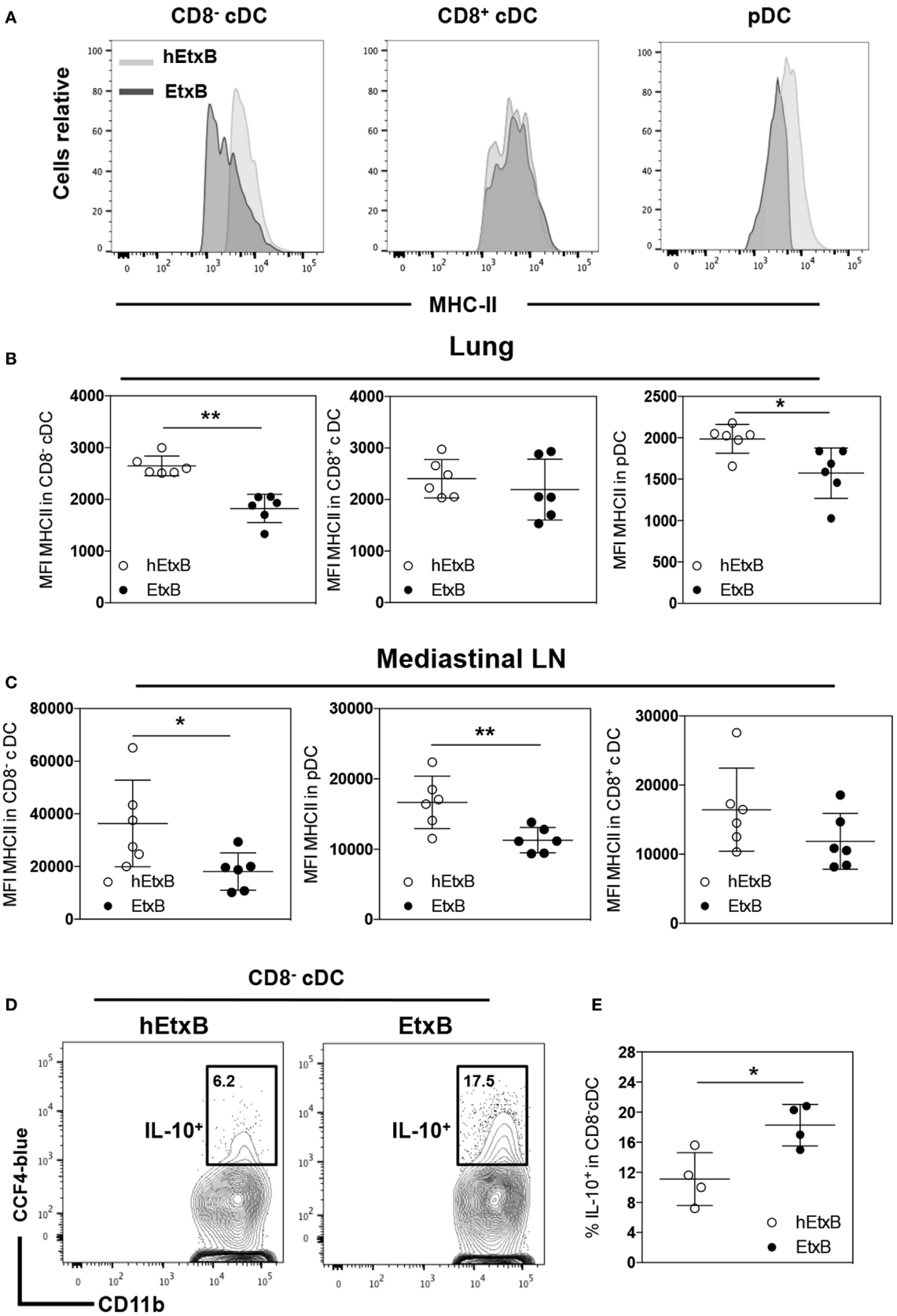

FIGURE 4 | **Continued**

FIGURE 4 | Continued
EtxB promotes immature dendritic cells (DCs). C57BL/6 mice were treated intranasally with 100 μg/mouse EtxB or heat-EtxB (hEtxB) for 3 days. On day 2.5 posttreatment, lung and mediastinal lymph node (mLN) were removed and analyzed. Flow cytometric histograms **(A)** and dot plots of membrane expression of major histocompatibility complex (MHC) class II on plasmacytoid DC (pDC, $CD11c^{low}CD11b^{-}CD8^{-}$MHC-II^{+}), $CD8^{-}$, and $CD8^{+}$ conventional DCs ($CD8^{+}$ or $CD8^{-}$ cDC, $CD11c^{high}CD11b^{+}CD8^{-/+}$ MHC-II^{+}) in the lung **(B)** and mLN **(C)**. ITIB mice were administered either 100 μg/mouse EtxB or hEtxB for 3 days. Flow cytometric contour plots **(D)** and dot plots **(E)** represent the frequencies of IL-10^{+} (CCF4-blue) $CD8^{-}$ cDC cells in the lung 1 day posttreatment. In panel **(A–C)**, results are from one experiment representative of six C57BL/6 mice per group from three independent experiments. In **(D,E)**, results are from one experiment representative of four ITIB per group from two independent experiments. In dot plots, the mean and SD are represented and each circle represents one mouse: white dots hEtxB-treated animals, black dots EtxB-treated animals. $^{*}p < 0.05$ and $^{**}p < 0.005$ compared with hEtxB-treated mice (Mann–Whitney *U*-test).

on transgenic T cells, but that BMDCs treated with EtxB were unable to induce T cell expression of CD69 above background levels (**Figures 5D,E**). Overall, these findings indicate that EtxB directly promotes *in vitro* "immature" BMDCs that fail to activate naïve $CD4^{+}$ T cells.

DISCUSSION

EtxB has been shown to ameliorate immune pathology in numerous mouse models of inflammatory disease (23–25). However, the way EtxB alters the immune system to mediate these effects has not been fully elucidated. Here, we show that EtxB acts directly at mucosal sites to increase the proportion and function of suppressive Treg cells and to promote $CD8^{-}$ cDCs with an immature phenotype. Importantly, EtxB is able to act directly on BMDCs to promote immature antigen-presenting cells that do not have the capacity to activate naïve $CD4^{+}$ T cells. Together, these data indicate that the immunoregulatory effects of EtxB work by increasing the frequency of leukocytes that promote tolerance, and further modulating their function to dampen T cell activation.

Our results build substantially on previous studies indicating that i.n. administration of EtxB increased the frequency of $Foxp3^{+}$ Treg cells (24, 26). In our study, we confirm that EtxB increases Treg proportion and show that this occurs rapidly and preferentially in mucosal tissues. The data show that the likely cause of increased Treg cell frequency following EtxB treatment is due to increased proliferation of thymic-derived Treg cells, with a concomitant decrease in cell death. While the molecular mechanism behind this cellular phenotype is unknown, studies on other cell types may provide some insight into the potential cause. It has been reported that EtxB–receptor interaction on B cells leads to induction of phosphatidylinositol-3 kinase (PI3-kinase)-dependent signaling cascades that regulate B cell activation (27). Because of the central role of PI3-kinase in cell growth, differentiation, survival and proliferation in T cells, and important role in Treg cell function (44), one can speculate that a mechanism dependent on PI3-kinase activation could contribute to increase proliferation and survival of Treg cells driven by EtxB.

Our results demonstrate that mucosal administration of EtxB promotes Tregs cells with a greater suppressive capacity. There are numerous mechanisms used by Tregs cell to impart suppression, including production of inhibitory cytokines (TGF-β, IL-10, IL-35), inhibitory receptors (CTLA-4, lymphocyte-activation gene 3), cytotoxicity (Granzyme/Perforin) and metabolic disruption (IL-2 deprivation-mediated apoptosis) (45). Blockade of CTLA-4 results in autoimmune disease and colitis in normal mice (46, 47), exacerbates diabetes in diabetes-prone non-obese mice (48), and abrogates Treg cell-mediated suppression (47, 49). Although, previous works suggested that the Treg cells that develop in absence of CTLA-4 have acquired compensatory suppressive mechanisms through enhanced TGF-β- or IL-10-dependent pathways (49, 50), one study revealed a non-redundant role for CTLA-4 expression by Treg cells to limit lymphopenia-induced CD4 T-cell expansion *in vivo*, which seems to be independent of IL-10, IL-35, TGF-β, or IDO (51). However, despite the important role of CTLA-4 for suppressive capacity, IL-10 is also important for Treg function, particularly at mucosal surfaces (52). Here, we observe that EtxB administration increases expression of IL-10 by Treg cells and we show that EtxB treatment results in increased in CTLA-4 expression. This results in enhanced functional capacity of Treg cells from EtxB-treated mice.

It has been previously demonstrated that EtxB treatment alters the innate immune system by reducing the proportion of precursors of cDCs and myeloid cells (32). Our work confirms this result in the lung and further shows that surface expression of the costimulatory ligand CD80 is upregulated on these cells. DCs are sentinels of the immune system and play an essential role in the maintenance of immune tolerance (14, 53). The potential of DCs to induce regulatory responses could be directly related to their maturation status (54). T cell inactivation in the lung can be induced by immature DCs that express low surface levels of MHC class II and costimulatory ligands. Notably, immature DCs are characterized by increased expression of programmed death-ligand 1, decreased expression of MHC class II, and decreased expression of costimulatory molecules (such as CD86 or CD40) (55–57). These "immature" DCs have the capacity to induce or expand Tregs cells (14). We observed that lung $CD8^{-}$cDC and pDC have significantly reduced membrane expression of MHC class II after EtxB treatment, consistent with an immature phenotype. In addition, we show that EtxB-treated mice had a significantly increased percentage of IL-10^{+} $CD8^{-}$cDC in the lung compared to hEtxB-treated mice. Taken together, this suggests that following mucosal EtxB administration, the DCs with an immature phenotype are promoted; further, these cells produce IL-10 locally that may play a role in the establishment of an immunoregulatory microenvironment in the lung. In the mLN, EtxB treatment has no effect on Treg cells and DCs precursors at day 2.5 following i.n. administration. However, we observed increase proportion of immature $CD8^{-}$cDC and pDC. Interestingly, although expression of CCR7 is considered an indicator of activated DCs, some "immature" DCs in peripheral tissues such as the lung can also up regulate CCR7, which allows them to migrate to the secondary lymphoid organs (58).

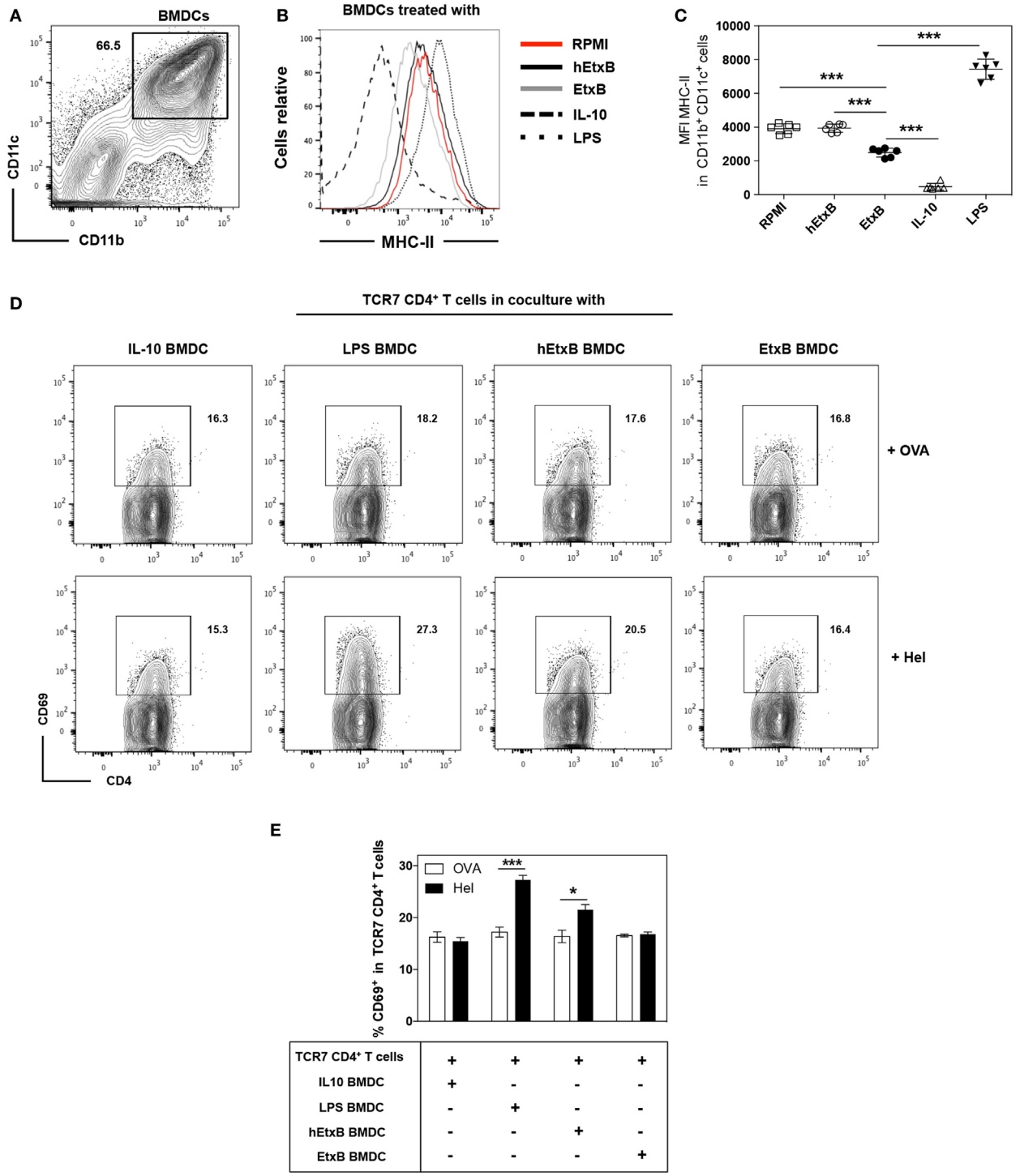

FIGURE 5 | **EtxB inhibits the T cell activation capacity of bone marrow-derived dendritic cells (BMDCs) *in vitro***. BMDCs were induced by culturing bone marrow cells of C57BL/6 mice cultured with GM-CSF, IL-4 and 2-Mercaptoethanol for 6 days. **(A)** Flow cytometric contour plot of CD11b/CD11c expression on BMDC cultures. **(B,C)** Membrane expression of major histocompatibility complex (MHC) class II on BMDC cultures from different conditions as indicated, BMDCs were defined as CD11b$^+$ CD11c$^+$ cells. **(D)** Flow cytometric contour plots and bar chart **(E)** of CD69 expression on naïve TCR7 CD4$^+$ T cells co-cultured with BMDCs generated under the indicated conditions, pulsed with either hen egg lysozyme (HEL) or ovalbumin (OVA) protein and then fixed. In dot plots, the mean and SD are represented and each symbol represents a technical replicate: white squares RPMI only treated cells, white circles hEtxB-treated cells, black circles EtxB-treated cells, white triangles interleukin-10 (IL-10)-induced tolerogenic BMDCs, black triangle lipopolysaccharide (LPS) treated BMDCs. In the bar chart, the height of the bar represents the mean; the error bars the SD. Results are from one experiment representative of three independent experiments. *$p < 0.05$ and ***$p < 0.0005$ (Mann–Whitney U-test).

In this study, we have addressed the question of how administration of EtxB induces an immunoregulatory microenvironment in the lung. Our results suggest that EtxB is able to promote Treg cells and "immature" DCs. In both cases, there is a marked increase in IL-10 production. Interestingly, IL-10 is able to support the induction of both immature DCs and Treg cells (14, 59). Previous studies have shown increased expression of *Il10* transcript in epithelial cells and $CD11b^+$ cells following EtxB treatment (25, 26). Taken together, IL-10 production after EtxB administration is likely one of the key mechanisms supporting the increase in Treg cells and immature DCs. Interestingly, ERK1/2 pathway is one of the signaling cascades that is activated in macrophages and DCs that results in IL-10 expression (60). Of note, Polumuri et al shown that TLR4 engagement in murine innate cells activates the PI3-kinase/Akt pathway and promotes IL-10 production that is reversed by PI3-kinase inhibition (61). Because EtxB induces PI3-kinase and MAPK/ERK kinase signaling cascades in B cells, it would be interesting to assess the potential link between theses pathways and IL-10 production in Treg and DCs following EtxB administration. Also, how EtxB directly or indirectly promotes Treg cells and immature DCs able to produce IL-10 warrants further study.

Taken together, our study demonstrates that EtxB exerts its effects *in vivo* mainly at mucosal surfaces. It limits T cell activation through two mechanisms: first through increasing "immature" IL-10^+ DCs that cannot activate T cells and second through increasing the proportion and function of Treg cells that limit T cell expansion. This model of EtxB action could explain why mucosal administration of EtxB protects from different T cell-dependent autoimmune diseases (23, 25) and suggests mucosal administration of EtxB as attractive therapeutic treatment for inflammatory disorders.

ETHICS STATEMENT

All experiments were performed according to the regulations of the UK Home Office Scientific Procedures Act (1986) under the UK Home Office license PPL 80/2526.

AUTHOR CONTRIBUTIONS

AB conceived, designed the study, and performed the experiments and wrote the manuscript. AW contributed to study design and reviewed the manuscript. ML conceived and designed the study and wrote the manuscript.

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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.2017.00560/full#supplementary-material.

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REVIEW
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From IgG Fusion Proteins to Engineered-Specific Human Regulatory T Cells: A Life of Tolerance

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Recent efforts have concentrated on approaches to expand and "specify" human regulatory T cells (Tregs) and to apply them to modulate adverse immune responses in autoimmunity and hemophilia. We have used retroviral transduction of specific T-cell receptor, single chain Fv, or antigen domains in Tregs to achieve this goal. Each of these approaches have advantages and disadvantages. Results with these engineered T cells and evolution of the research developments and paths that led to the development of specific regulatory approaches for tolerance are summarized.

Keywords: regulatory T cells, chimeric antigen receptor, engineered T cells, hemophilia A, multiple sclerosis, B-cell receptor, single-chain variable fragment

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INTRODUCTION

Self-non-self discrimination, i.e., immunologic tolerance, is a hallmark of the immune system. Implicit in this paradigm is specificity. Understanding how the immune system learns what is self can be demonstrated by a conversation from Sir Arthur Conan Doyle's short story "Silver Blaze" about a murder that took place in the stable of this prize racehorse:

"Is there any other point to which you wish to direct my attention?" asked Dr. Watson.
"To the curious incident of the dog in the night time!"
"But the dog did nothing in the night time."
"That," remarked Sherlock Holmes, "was the curious incident." (1)

As insightful as ever, the master detective realized that the watchdog in the stable recognized the culprit as "familiar" and thus did not respond. The watchdogs of the immune system, the T and B lymphocytes, also must learn what self (familiar) is and what is not (foreign) in order to provide specific responses to potential dangers. Immunologic tolerance must be learned (2, 3). This property of the immune system has driven research in my lab for decades, most recently in the area of specific regulatory T cells (Tregs). In this review, I will summarize the research that led to the development of specific Tregs to induce tolerance and reverse adverse immune responses.

Much of the early work was pioneered by the late Weigle and colleagues (4–6) with IgG as a tolerogen and extended by seminal studies from Yves Borel, who used IgG fusions as tolerogens (7, 8), the latter being shown to depend on the presence of the IgG Fc fragment (9, 10). Later, we used gene therapy of B cells expressing fusions of antigens with an IgG heavy chain to be highly tolerogenic in several systems (11–14) and showed that this approach was dependent on Tregs for both its induction and maintenance (15–17). Indeed, recent development of Fc fusions of clotting factors like Factor VIII (FVIII) and FIX, designed for a longer half-life *in vivo* (18–20),

have turned out to be tolerogenic and to induce Tregs (21, 22), as discussed below. This is supported by anecdotal cases in hemophilia A patients that suggest that FVIII-Fc is potentially tolerogenic (23–25), which is leading to a more highly powered clinical trial (26). The reason that Fc fusions are tolerogenic is not precisely known, but may involve regulatory epitopes in the constant region (27, 28) that turn on Tregs, and/or inhibitory Fc receptors (29).

In this review, we will summarize the evolution of the research paths that led to the development of specific Treg approaches for tolerance. We have concentrated recently on efforts to expand and "specify" Tregs (30, 31) and apply them to modulate adverse immune responses in autoimmunity and hemophilia.

Hemophilia A

Hemophilia A is an X-linked bleeding disorder caused by mutations in the FVIII (*F8*) gene. This gene encodes a 250 kDa protein, FVIII, which is a critical component of the blood coagulation cascade. Severe hemophilia A results from major deletions or inversions in the *F8* gene, such that these individual have less than 1% FVIII activity; mild hemophilia can occur with missense mutations, for example, that also lead to significantly reduced clotting efficacy. These disorders can be treated with recombinant or plasma-derived FVIII replacement therapy, either prophylactically or on demand. Unfortunately, a large subset of those receiving replacement FVIII develop an antidrug antibody response because they never developed tolerance to this human protein (unlike the dog in the nighttime!) In the hematology community, these antibodies are referred to as "inhibitors" because they can inhibit or neutralize the therapeutic function of FVIII, rendering this life-saving treatment ineffective. Inhibitor formation requires $CD4^+$ T cell help as evidenced originally in HIV-infected patients with inhibitors whose titers dropped when their T-cell levels diminished, but whose antibodies returned upon multi-drug therapy (32, 33). Further studies in a murine model (FVIII knockouts) verified this T-cell dependence (34, 35). Most of the inhibitory antibodies are directed at the A2 and C2 domains of the FVIII protein, which are critical for binding to partners in the cascade.

For several decades, the standard treatment in patients that develop inhibitors is repeated, high-dose FVIII therapy to reduce or eliminate titers, a process referred to clinically as "immune tolerance induction." This is an expensive process and does not work for all inhibitor cases, being successful primarily in patients with low-titered antibodies. Thus, we have targeted the A2 and C2 domains of the FVIII protein in our approaches for inducing tolerance to FVIII (13, 22). This would be important to achieve in inhibitor positive patients or to prevent inhibitor responses, in the first place, which is of clinical importance.

Fc FUSIONS IN HEMOPHILIA AND OTHER DISEASE MODELS

As noted above, IgG carriers have been shown to be highly tolerogenic. In part, this may reflect their long half-life in the circulation and even in tissues. In addition, binding to Fc receptors on B cells can deliver a negative signal that aborts full signaling (36). Teleologically, it is important that the immune system be tolerant of its own products. Immunoglobulins express an enormous range of specific receptors (idiotypes) that must be tolerated as their numbers increase and diversify during an immune response. Based on the hypothesis that IgG was a highly tolerogenic carrier, we devised a strategy to express a variety antigens in frame on an IgG heavy chain scaffold. Recombinant expression of these fusion proteins was predicted to be tolerogenic, and indeed they were (22, 37). We also reasoned that retroviral expression in B cells in which the fusion heavy chain would be assembled with endogenous light chains would lead to secretion of hybrid molecules into the circulation to tolerize the autologous host. Indeed, this also occurred (11). However, this was not due to the secreted product, but rather by B-cell tolerogenic presentation (38), confirming the work of Eynon and Parker (39) and Fuchs and Matzinger (40). Importantly, we found that B-cell expression of MHC class II and B7, but not Fc receptors on the transduced B cells was required (41–43), and that the IgG scaffold enhanced the tolerogenicity of these cells (44). Further data suggested that IgG may contain tolerogenic epitopes (27).

Over the next decade, we utilized this system to induce tolerance to a variety of antigens in multiple autoimmune disease models (uveitis, EAE, diabetes, arthritis) and in hemophilia A (12, 13, 15, 45–48). In many of these studies, a role for Tregs was suggested or demonstrated for the induction or maintenance of tolerance (16, 47). Thus, we embarked on an effort to develop a platform for Treg-based tolerance protocol, focusing on two different diseases, hemophilia and multiple sclerosis (MS). In the former, an adverse (T-cell dependent) antibody response blocks effective therapy, whereas in the latter, T-cell-mediated pathology in the central nervous system is the target.

RATIONALE FOR DESIGNING SPECIFIC Tregs

Polyclonal human Tregs have been proposed to treat autoimmune diseases and transplant rejection, as well as to suppress undesirable immune responses to bio-therapeutics such as recombinant or plasma-derived FVIII. Several of these are already in clinical trials (49–51). While these appear to be safe, they are polyclonal T cells that include a diverse repertoire of specificities and large numbers of polyclonal Tregs are needed. Thus, there is the possibility that non-specific immunosuppression and viral reactivation could occur (52). Moreover, the frequency of relevant specific Tregs is quite low in a normal repertoire. One could attempt to enrich and expand Tregs using antigen and/or tetramers in the presence of antigen-presenting cells (APC) and IL-2, as long as they do not revert to an effector pathogenic phenotype.

We elected instead to render human Tregs specific, based on chimeric antigen receptor (CAR) therapy for cancer (53–55), and to maintain their functional properties during expansion with a novel approach (56). Hence, we engineered specificity into polyclonal Tregs *via* retroviral transduction of specific

T-cell receptors (TCR) or CARs [single-chain variable fragment (scFv)], or even antigen [B-cell antibody receptor (BAR)].

FOUR FLAVORS OF SPECIFIC Tregs

TCR-Transduced CD4 T Cells

Inspired by the success of engineered cytotoxic CAR T cells in blood cancers (55), our goal was to apply this approach to directly create large numbers of specific Tregs with engineered receptors. As noted above, based on our experience with retroviral transduction of Fc fusions into activated B cells, we had established a role for Tregs in the tolerance so induced. The buffy coat fractions in all of the experiments to be described below were from peripheral blood mononuclear cells (PBMC) from healthy normal adult donors from the American Red Cross or the NIH Blood Bank. CD4 fractions were isolated by magnetic cell enrichment, then labeled, and sorted based upon the following cell surface markers: naïve CD4 effector T cells were $CD4^+$, $CD25^-$ $CD127^+$, and $CD45RA^+$ and Tregs were $CD4^+$, $CD25^{high}$, $CD127^{low}$ (and the latter were Foxp3 and Helios positive, reflecting their status as "natural" Tregs).

In collaboration with Dr. Kate Pratt, who had obtained multiple clones of FVIII-specific T effectors from patients with hemophilia A, we determined the TCR variable (V) region genes from two of these clones, termed 17195 and 171911. In the first iteration for specific CD4 effectors and Tregs, retroviral vectors were engineered to express the 17195 or 171911 TCR variable regions in polyclonal T cells activated initially with anti-human CD3. The transduced T cells were expanded as described by Kim et al. (30) with irradiated PBMC's as APC. Notably, Tregs were expanded but their cultures also contained random oligonucleotides (ODNs), which Kim et al. (56) had shown serve to maintain Treg properties (Foxp3 and Helios). **Figure 1** illustrates the principle.

With this approach, we obtained large numbers of expanded FVIII-specific T cells expressing the 17195 or 171911 TCRs that we demonstrated were highly reactive to the FVIII peptides, albeit with different affinities based on the affinity of the initial clones (57). The transduced T effectors proliferated and produced cytokines in response to the FVIII peptide (pC2, 2191–2210) on appropriate DR1 APCs just as effectively as anti-CD3 stimulation of the donors; moreover, specific antigen led to an expansion of the cells expressing the TCR as evidenced by tetramer binding (30). Transduced and expanded Tregs also responded to peptide and displayed increased levels of Foxp3, Helios, GARP, and LAP, typical of activated Tregs, but did not produce significant levels of IL-2 and interferon gamma (IFNγ). Thus, these cells looked like and smelled like human Tregs. We next tested whether they could suppress a FVIII-specific response and found that proliferation of FVIII-specific effector T cells was suppressed even when the effector cells were cultured at an 8:1 ratio to Tregs (30).

As noted above, the antibody response to FVIII in hemophilia A patients is a major hindrance to effective therapy for bleeding. Therefore, we have tested the effect of engineered FVIII-specific human Tregs on an *in vitro* recall antibody response to FVIII in humanized (DR1) hemophilic knockout mice, using the approach of Hausl et al. (58). Despite being a xenogeneic system, the engineered Tregs were able to suppress the recall antibody response to FVIII (30). Interestingly, although the engineered TCR recognizes a single peptide in a large protein, the antibody response to other major epitopes of FVIII was also suppressed. This indicates that bystander suppression of other T (and B) cells had occurred *in vitro.* Subsequently, we demonstrated that this could also occur *in vivo* so it was not due to a culture artifact (31). Thus, we have engineered specificity into expanding human Tregs and shown that they can suppress the antibody response to FVIII effectively.

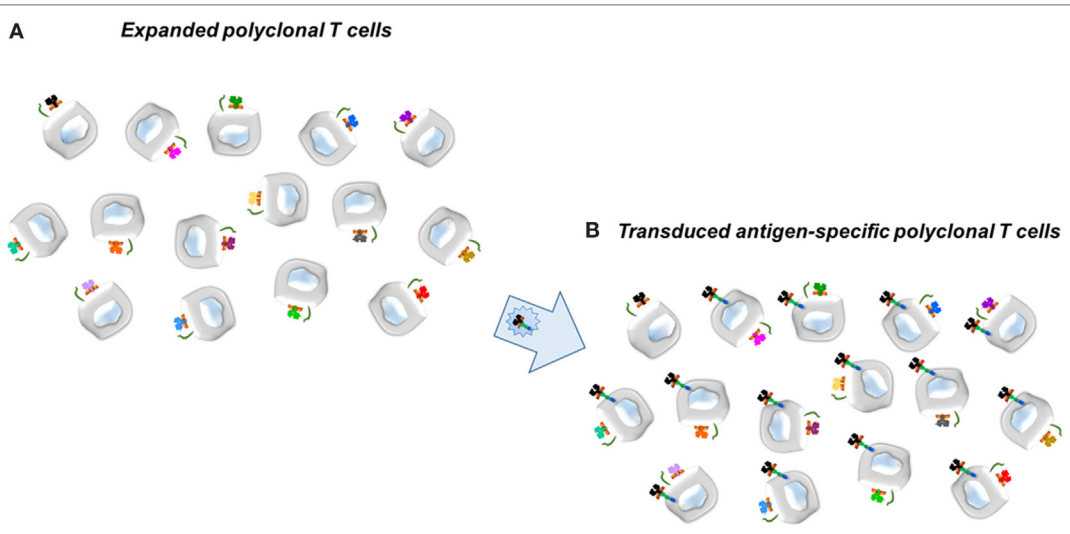

FIGURE 1 | Scheme for transduction of Factor VIII-specific (T-cell) receptors into expanded human T cells, either $CD4^+$ effector or $CD25^{hi}$ regulatory T cells. **(A)** Expanded polyclonal T cells. **(B)** Transduced antigen-specific T cells. Retroviral vector to express the T-cell receptors, for example, is shown in the large arrow.

scFv Transduced CD4 T Cells

While these TCR-transduced Tregs were highly effective, they are MHC class II restricted, thus limiting their eventual utility to patients sharing the same MHC globally. Therefore, in collaboration with Anja Naumann Schmidt and Christoph Königs in Frankfurt, we developed a second approach to engineer specificity, namely a scFv, as shown in **Figure 2**. Dr. Schmidt used phage display to obtain a number of single chain antibodies that reacted with different domains of FVIII (59, 60). One of these, called ANS8, recognized the A2 domain of FVIII. This scFv was incorporated into our retroviral vector and used to transduce both CD4 effectors and Tregs. These scFv transduced cells recognized free FVIII but responded to membrane or plate bound FVIII more effectively (31), presumably reflecting the exposure of the A2 domain under these conditions.

ANS8 CAR human Tregs were generated and tested under the same conditions as the 17195 TCR Tregs. These Tregs also suppress the proliferation of FVIII-specific T effector cells, but most importantly suppressed the antibody response to FVIII both *in vitro* and *in vivo* (31). Notably, both the ANS8 CAR-transduced Tregs and 17195 (TCR)-transduced Tregs were effective in these assays at effector: target ratios with effector cells in excess (31). Suppression of the antibody response by these human Tregs *in vivo* lasted up to 8 weeks. When these mice were boosted with FVIII at 8 weeks post immunization, suppression was lost presumably because the human cells were rejected by the immunocompetent murine hosts. Nevertheless, these data demonstrate that both CAR- and TCR-transduced specific Tregs that recognize different B-cell and T-cell domains of FVIII can be suppressive against multiple epitopes of this large immunogenic protein. Despite this bystander effect, the response to an unrelated antigen (TNP-sheep RBC) was not affected. Thus, suppression in this model is specific.

"BAR" Expressing CD4 Tregs and Cytotoxic T Cells

We recently applied the principle of engineered cytotoxic CAR T cells to directly target FVIII-specific B cells. In lieu of a chimeric antibody, we engineered immunodominant B-cell domains of FVIII into both expanded cytotoxic CD8 and regulatory CD4 T cells (**Figure 3**). The principle hypothesis was that FVIII-specific B cells possess IgM and IgD receptors that recognize FVIII conformational epitopes. When they would encounter engineered cytotoxic T cells, for example, they would bind these epitopes to form a synapse and would receive a putative negative signal from these cytotoxic cells. This was recently demonstrated by Ellebrecht et al. (61), who used engineered cytotoxic T cells expressing a major skin target (desmoglein 3) in pemphigus vulgaris, a devastating skin disease. They showed that human cytotoxic T cells expressing desmoglein 3 could kill B-cell hybridomas specific for desmoglein. To apply this for hemophilia, we engineered immunodominant C2 or A2 domains (that are the major targets of inhibitory antibodies to FVIII into both human and mouse cytotoxic cells). These BAR expressing cytotoxic T cells were able to kill C2- and A2-specific hybridomas *in vitro* and *in vivo*. Moreover, their specificity for FVIII-specific B cells was formally demonstrated in two additional assays: elimination of naïve B cells stimulated with polyclonal B-cell activator, LPS, and inhibition of the antibody response to FVIII *in vivo*. Because they are domain-specific and did not display a bystander effect, both C2- and A2-BARs were needed to eliminate the response to full-length FVIII (62).

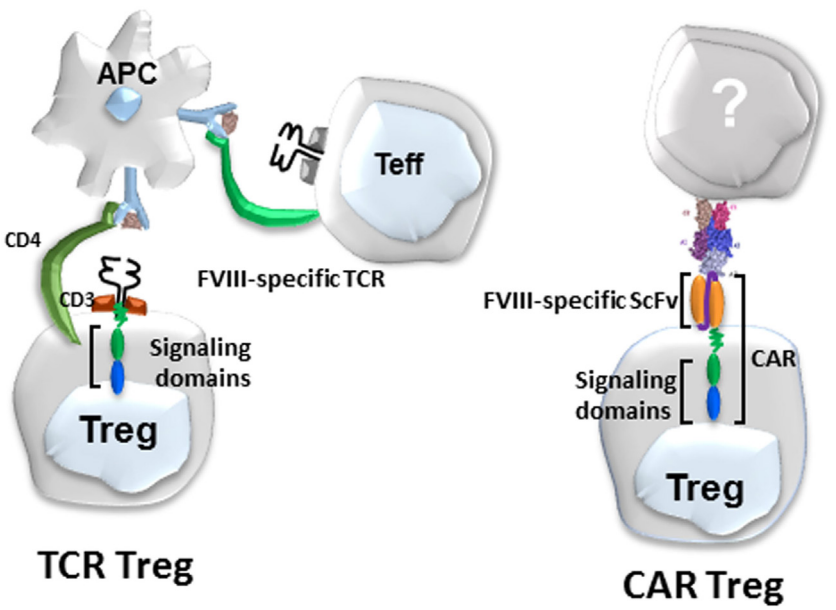

FIGURE 2 | Diagram of Factor VIII (FVIII)-specific T-cell receptors (TCR)-transduced (left) and single-chain variable fragment (scFv)-transduced human regulatory T cells (Tregs) (right).

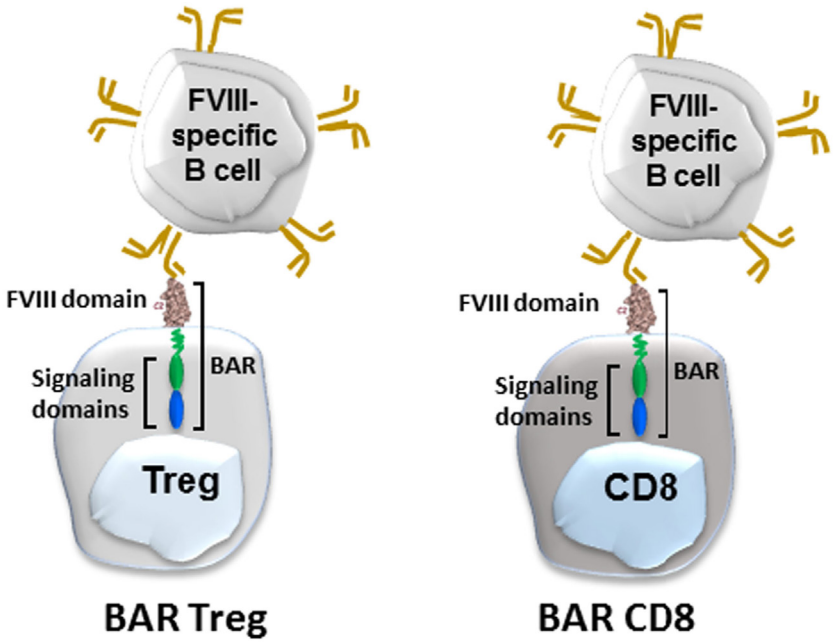

FIGURE 3 | Diagram of BAR-transduced regulatory T cells (Tregs) (left) and BAR-transduced cytotoxic CD8 T cells (right). BAR stands for B-cell antibody receptor, which in this case is a Factor VIII (FVIII) domain. The BCR (gold) binds the antigen on the Treg or cytotoxic T cells *via* its variable regions, which signals the T cell.

What about BAR Tregs? Theoretically, BAR-expressing Tregs could also interact with specific B cells, but we did not know whether they could directly or indirectly inhibit the B-cell response to FVIII. These data demonstrated that injections of human BAR Tregs into hemophilic mice did indeed prophylactically prevent the antibody response to FVIII (63). To examine the mechanism of this inhibition, we purified B and T cells from BAR tolerized and control mice and then performed classic mixing experiments. These results suggested that B cells may be directly targeted by BAR Tregs since they could not be "helped" by control non-tolerant T cells (64). Whether this is due to anergy or cytotoxicity of targeted B cells is under investigation.

DISCUSSION

Specific tolerance induction to treat a variety of adverse immune reactions is preferable to non-specific immune suppression. We have focused on the use of engineered specific Tregs and cytotoxic T cells and have developed four different approaches for applications to treat adverse immune responses in both monogenic diseases, like hemophilia (30, 31), as well as in autoimmunity. The notion of engineering specificity into T cells was pioneered by Eshhar (54, 65) and colleagues with an approach that he termed "T-bodies." Subsequent application of engineered cytotoxic T cells in the treatment of hematologic cancers has revolutionized therapy for those diseases (55, 66). Recently, several others have engineered T-Regs targeting different antigens (67–69). The most analogous to our studies are those of MacDonald et al. (68), who utilized a single chain Fv that targeted the HLA class I antigen, A2. We have used retroviral expression in human T-Regs of specific TCRs and an scFv that recognize FVIII T- and B-cell epitopes, respectively, for hemophilia, as well as antigen domains that would be recognized by B cells, all of which were functionally stable and competent to suppress FVIII responses *in vitro* and *in vivo*. In addition, we have extended this approach with a myelin basic protein-specific TCR to suppress autoimmune responses in a model for MS (70).

The mechanism(s) of suppression are not fully understood. Recent data suggest that signals from IL-2 derived from effector cells "turn on" a program of suppression by the engineered Tregs, and that this leads to a bystander effect in the local milieu. Further characterization of the mediators is in progress.

Determination of which kind of engineered Tregs will be most applicable will depend in part on the target antigen(s) and the disease entity and effector targets. The process described herein is a personalized medicine that could be limited to autologous donors. Given this limitation, as well as HLA restriction and the possibility that Tregs may be defective in certain diseases (71, 72), we envision that a generic/universally applicable population of Tregs can be prepared in which CRISPR/Cas9 technology can be used to remove endogenous receptors and MHCII to avoid graft-versus-host and allorecognition, respectively (73).

Future studies in larger animal species, such as dogs with hemophilia (74, 75), are planned as a step toward translation in human clinical studies.

AUTHOR CONTRIBUTIONS

DS is solely responsible for the content of this article.

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Innovative Approaches for Immune Tolerance to Factor VIII in the Treatment of Hemophilia A

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Hemophilia A (coagulation factor VIII deficiency) is a debilitating genetic disorder that is primarily treated with intravenous replacement therapy. Despite a variety of factor VIII protein formulations available, the risk of developing anti-dug antibodies ("inhibitors") remains. Overall, 20–30% of patients with severe disease develop inhibitors. Current clinical immune tolerance induction protocols to eliminate inhibitors are not effective in all patients, and there are no prophylactic protocols to prevent the immune response. New experimental therapies, such as gene and cell therapies, show promising results in pre-clinical studies in animal models of hemophilia. Examples include hepatic gene transfer with viral vectors, genetically engineered regulatory T cells (Treg), *in vivo* Treg induction using immune modulatory drugs, and maternal antigen transfer. Furthermore, an oral tolerance protocol is being developed based on transgenic lettuce plants, which suppressed inhibitor formation in hemophilic mice and dogs. Hopefully, some of these innovative approaches will reduce the risk of and/or more effectively eliminate inhibitor formation in future treatment of hemophilia A.

Keywords: factor VIII, hemophilia A, immune tolerance, regulatory T cell, oral tolerance, rapamycin, gene therapy, AAV vectors

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INTRODUCTION

Hemophilia A, or factor VIII (FVIII) deficiency, is one of the most prevalent genetic bleeding disorders, which affects 1:5,000 male births. It is inherited as an X-linked recessive trait, although it can also be acquired in advanced age as a result of autoimmunity, cancer, or various metabolic disorders affecting both males and females (1). Both inherited and acquired forms of hemophilia are manifested by frequent bleeding episodes, hemorrhages into the skin and body cavities, pain, orthopedic disability, and significant morbidity (2).

Classification and severity of hemophilia has been based on circulating levels and residual activity of coagulation factors in plasma. Residual factor activity levels of <1%, compared to normal plasma are classified as severe, 1–5% moderate, and 5–40% mild (3). Patients with large deletions in the F8 gene, or inversion mutation in intron 22 (I22I), have no circulating FVIII, suffer from severe hemophilia and are most likely to develop an adverse immune reaction to exogenous FVIII infusions (4). Patients with a missense or disruption mutation may express various amounts of non-functional or partially functional FVIII protein. Patients with milder forms of hemophilia A respond better to treatment and are at a lower risk of developing adverse inhibitory antibodies (5).

Factor VIII is a 280-kDa glycoprotein that circulates in the blood at ~200 ng/ml (where it is closely associated with von Willebrand factor) and, upon activation, serves as a co-factor to the serine protease factor IX (FIX), which catalyzes a critical step in the intrinsic pathway of the

coagulation cascade. FVIII is initially synthesized as a 2,351-aa polypeptide that is organized into A1–A2–B–A3–C1–C2 domains and processed into non-covalently linked heavy and light chains prior to secretion. Since FVIII is critical for the enzymatic function of FIX, mutations in either protein can cause the bleeding phenotype that is characteristic for hemophilia. The majority of hemophilic patients have mutations in their F8 gene (resulting in hemophilia A), while mutations in F9 result in hemophilia B.

It is estimated that up to 30% of patients with severe hemophilia A and 5% of patients with milder forms of the disease form anti-drug antibodies, termed "inhibitors." These inhibitors are detected and measured in the Bethesda assay, with 1 Bethesda unit representing 50% residual coagulation activity in normal plasma after incubation with a patient's test plasma. That fact that potent antibody responses occur despite FVIII being given intravenously at low antigen doses illustrates the immunogenicity of this protein. Patients with >5 BU/ml typically fail to respond to factor replacement therapy, requiring the use of bypass agents. Recent clinical studies have demonstrated that genetic variables and F8 gene mutation type are important determinants of an individual's risk for inhibitor formation, as is ethnicity and intensity of early treatment (6). While clinical protocols have been available to restore hemostasis in inhibitor patients and to reverse the inhibitor response, these methods are suboptimal, expensive, and not successful in all patients. Moreover, there are no prophylactic protocols to prevent inhibitor formation. These limitations have fueled recent diverse pre-clinical developments of alternative strategies for immune tolerance induction (ITI) to FVIII, which are based on emerging technologies such as gene therapy, regulatory T-cell (Treg) therapy, and transgenic crop plants for oral tolerance, among others (7).

CURRENT AND FUTURE TREATMENTS OF HEMOPHILIA AND THEIR IMMUNE IMPLICATIONS

Currently, hemophilia A is treated with an intravenous infusion of plasma-derived or recombinant clotting FVIII concentrates, which can be on demand or prophylactic (8, 9). However, the half-life of infused FVIII concentrate is very short, only 10–12 h, and thus, it must be administered frequently, an inconvenience for the patient. Also, these frequent infusions create the possibility of introducing infections through the indwelling catheter as well as a risk of micro bleeds. Therefore, the development of longer acting FVIII concentrates became the next step in clinical care for HA patients (10). Longer lasting, or extended half-life clotting factors have been recently introduced for therapeutic and prophylactic treatment of hemophilia A. These include Fc and albumin fusion proteins as well as PEGylated FVIII (11–15). However, half-life extension of these products has been modest (more than twofold increase) (10, 16). Based on older observations on tolerogenic effects of immunoglobulin conjugation, it is hoped that Fc-FVIII may have reduced immunogenicity, which is supported by some pre-clinical data (17). Clinical evaluation in previously untreated patients (PUPs) should answer this question.

As opposed to generating less immunogenic FVIII molecules or employing ITI, an alternative strategy to avoid the effects of inhibitors against FVIII altogether is to develop bypassing agents that promote coagulation through pathways that either do not require FVIII or that mimic the function of FVIII. Novel drugs that fall into this category include Emicizumab (Chugai Pharmaceutical, Chuo, Tokyo, Japan), a human monoclonal bi-specific antibody, which is administered subcutaneously once per week (18, 19) and binds to both activated coagulation FIX and FX, mimicking the function of FVIII (20). Fitusiran is an experimental RNAi-based drug developed by Alnylam Pharmaceuticals (Cambridge, MA, USA) that targets endogenous anticoagulant antithrombin expression in the liver (21). As a result, Fitusiran improves homeostasis by promoting thrombin generation. Both drugs are currently undergoing extensive clinical testing.

Rather than treating hemophilia with more or less frequent drug administrations, gene therapy has the potential to cure the disease. Multiple Phase I/II clinical trials are testing hepatic *in vivo* gene transfer with adeno-associated viral (AAV) vectors in patients with severe hemophilia A, in some cases achieving normal FVIII levels (22). In pre-clinical large animal studies, sustained expression for >1 decade had been observed with this approach. While FVIII is normally produced by liver endothelial cells, these gene therapies target transgene expression to the more abundant hepatocytes. In these trials, patients must have demonstrated extensive prior treatment with FVIII protein without having formed inhibitors. Nonetheless, a large body of studies in animal models of hemophilia has demonstrated the potential of hepatic gene transfer to induce immune tolerance to the transgene product, which is discussed in further detail below.

INHIBITOR FORMATION AND CLINICAL ITI

In traditional intravenous FVIII replacement therapy, the appearance of inhibitors is usually observed in PUPs, i.e., young pediatric patients, during the first 50 days of exposure to FVIII (23). However, increased incidence of inhibitor development was also reported in older patients (50+ years), with previous exposure to FVIII (24). Inhibitor formation is a serious complication in the treatment of hemophilia. These antibodies make replacement therapy ineffective, thereby substantially complicating treatment, increase risks of morbidity and mortality, and substantially elevate costs of treatment. The mechanism of inhibitor formation is multifactorial and not entirely understood. Several predisposing risk factors have been identified. Genetic risk factors include F8 mutation types (such as large deletions, nonsense mutations, and intron 22 inversions), which are associated with a higher rate of inhibitor development. Patients of African-American and Hispanic ethnicity have a higher risk for inhibitor formation. Family and sibling history, major histocompatibility complex class II alleles, and polymorphisms

in immune regulatory genes coding for cytokines (IL-10, TNFα) and other molecules such as CTLA-4 are likely important contributors (25, 26). Other modifiers include production of indoleamine-pyrrole 2,3-dioxygenase (IDO) enzyme, inflammation, and age and intensity of first exposure to FVIII (27, 28).

B-cell activation, leading to inhibitor formation, is $CD4^+$ T-helper cell dependent, and several $CD4^+$ T-cell epitopes have been mapped in humans (29). Co-stimulation via CD80/86-CD28, CD40-40L, and ICOS-ICOSL pathways is required, which can be exploited for tolerance induction using co-stimulation blockers such as anti-CD40L/CTLA-4-IgG combination or anti-ICOS monoclonal antibody (30). A related strategy is based on interference with T-cell receptor (TCR) signaling using anti-CD3, which appears to favor induction of $CD4^+CD25^+FoxP3^+$ Tregs (31). Inhibitors target various parts of FVIII, although the A2 and C2 domains are believed to be the most immunogenic. Marginal zone macrophages have been found to be important for the capture and accumulation of FVIII in the spleen (32). Otherwise, remarkably little is known about the roles of professional antigen-presenting cells (APCs), such as dendritic cells (DCs) in the events that lead to FVIII-specific B-cell activation. Memory T cells may be re-activated by innate immune signaling through toll-like receptors 7 or 9 (33, 34).

Current clinical protocols for ITI are designed to eradicate inhibitors. The most commonly used form of ITI employs frequent (daily), high dose (up to 200 IU/kg/day) infusions of FVIII to eliminate inhibitors (35, 36). To date, there is no definitive mechanistic explanation as to how high doses of FVIII can induce tolerance. One of the proposed theories is that repetitive, high doses of antigen can suppress activated T-cell responses by overstimulation with antigen, followed by anergy and deletion (37). ITI also targets FVIII-specific memory cells and may assist in the induction of Treg (38). ITI is considered successful if inhibitor titers fall below 0.6 BU/ml, and FVIII function is normalized (39). Duration of ITI varies among patients from 9 to 48 months, according to the International Immune Tolerance Registry and the North American Immune Tolerance Registry. Therefore, ITI protocols often cost >$1M to complete. Outcomes of ITI therapies are variable as well. Only 50–70% of patients benefit from "traditional" ITI protocols. Some patients, who initially respond to ITI therapy, may experience anamnesis (inhibitor re-appearance) with repeated exposure to FVIII. Taking in consideration the high cost, moderate success rate, long duration, inconvenience of daily infusions, and a risk of anamnesis, ITI protocols can be modified to include other therapies and immunomodulation (**Figure 1**).

NOVEL APPROACHES TO IMMUNE TOLERANCE IN HEMOPHILIA—*IN VIVO* Treg INDUCTION VS Treg THERAPY

Over the past decade, strong evidence has emerged that Tregs are an integral part of immune tolerance to coagulation factors in gene and protein replacement therapies (40–42). It should therefore be possible to promote tolerance to FVIII by enhancing *in vivo* Treg induction or by development of a Treg-based cell therapy. Thymic-derived and peripherally induced $CD4^+CD25^+FoxP3^+$ Tregs are critical immune regulators to prevent autoimmune disease and for tolerance to "non-self" antigens. Treg may suppress immune responses via diverse mechanisms that include direct cell to cell contact, release of suppressive cytokine such as IL-10 and/or TGF-β cytokines, and modulation of APC maturation and function, thus preventing differentiation of T cells into effector cells and promoting their conversion into Tregs (43). One potent approach to shifting the balance from an effector T-cell to a Treg response to an exogenous protein is co-administration of the antigen with the mTOR inhibitor rapamycin (44) (**Figure 1**).

Cell cycle progression in activated T cells upon stimulation of the IL-2 receptor requires signaling through the mTOR pathway in conventional T cells. Activation of mTOR also promotes glycolysis, a metabolic pathway that effector T cells heavily depend on. Therefore, antigen presentation combined with blockage of the mTOR pathway by rapamycin results in apoptosis of activated T cells. However, Treg induction is enhanced in the presence of rapamycin. Treg heavily depend on IL-2 but preferentially utilize alternative downstream signaling pathways (via Stat5) and lipid metabolism, allowing these cells to expand in the presence of rapamycin. Hence, a 1-month oral regimen of rapamycin, combined with low-dose intravenous FVIII administration, resulted in long-term tolerance to therapeutic FVIII administration in hemophilia A mice (45). Antigen-specific tolerance was maintained for months after general immune suppressive effects had waned. This outcome correlated with the induction of FVIII-specific $CD4^+CD25^+FoxP3^+$ Treg. To further enhance efficacy and reduce systemic immune suppressive effects, rapamycin may be packaged into polymeric synthetic nanoparticles. Transient co-administration of FVIII and rapamycin-nanoparticles similarly induced lasting tolerance in the hemophilia A mouse model and also diminished pre-existing inhibitors (46, 47). The tolerogenic effect of rapamycin can be further enhanced by addition of cytokines such as IL-10 or Flt3L (44, 45, 48). Flt3L is widely used for *in vivo* expansion of DCs. Interestingly, in the presence of rapamycin (within a certain dose range), Flt3L selectively expands plasmacytoid DCs (pDCs), resulting in further increased Treg induction compared to antigen/rapamycin alone (48). In contrast to other DCs, pDCs uniquely express a microRNA (miRNA) that indirectly causes a more active mTOR pathway (48, 49). Hence, pDCs are more resistant to mTOR inhibition. Evidence has been presented that pDC enhances Treg induction through expression of IDO, which has effects on signal transduction but also catalyzes the first step in tryptophan catabolism, resulting in degradation products that may promote Treg induction (50).

An alternative method to enrich for Treg *in vivo* is the use of IL-2 complexed with a monoclonal antibody against IL-2 (IL-2/IL-2 mAb complexes), thereby aiding in the rapid expansion of $CD4^+CD25^+FoxP3^+$ Treg. Pretreatment with these complexes have been shown to produce activated and highly suppressive Treg in mice that prevented autoimmunity and showed long-term acceptance in transplant rejection studies (51). In hemophilia A mice, this regimen robustly suppressed inhibitor formation to either FVIII replacement therapy or plasmid-mediated gene

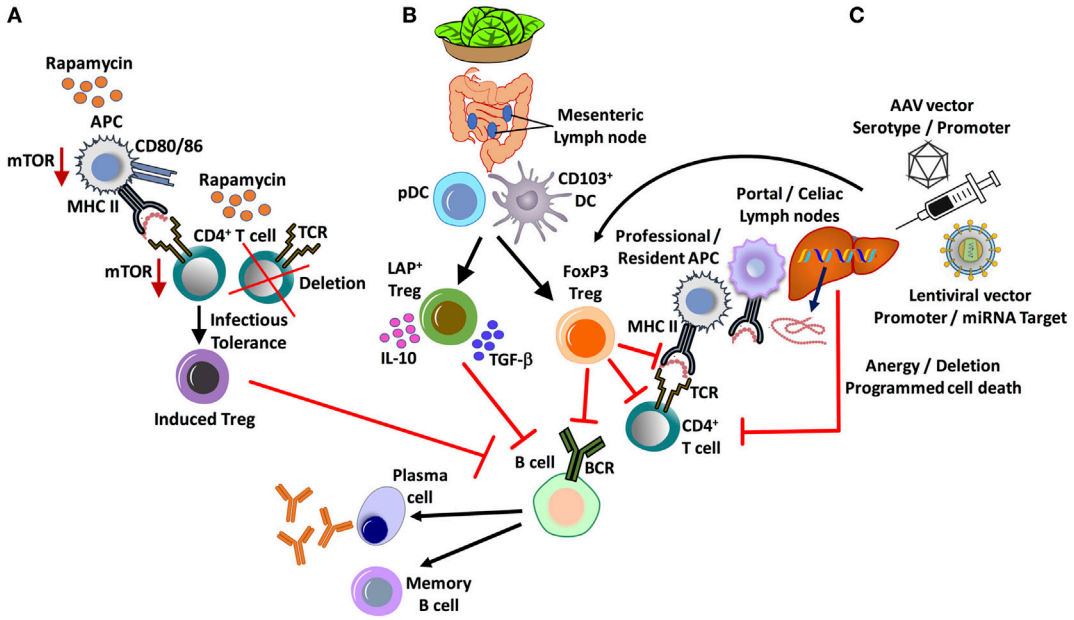

FIGURE 1 | Major *in vivo* approaches for tolerance induction and suppression of inhibitor formation in hemophilia. **(A)** Administration of the widely used clinical immunosuppressant, rapamycin, results in the selective deletion of $CD4^+$ T-helper cells and enrichment of $FoxP3^+$ regulatory T cell (Treg), exploiting differential use of the mTOR pathway. **(B)** Oral tolerance by lettuce encapsulated clotting factor leads to the suppression of inhibitors by at least two subsets of Treg: $CD4^+CD25^-LAP^+FoxP3^-$ and $CD4^+CD25^+FoxP3^+$ Treg. Antigen presentation by gut resident $CD103^+$ dendritic cell (DC), as well as plasmacytoid DCs (pDCs), supports Treg induction. **(C)** Hepatic gene transfer by adeno-associated viral (AAV) or lentiviral vector induces tolerance by multiple mechanisms, which include programmed cell death of $CD4^+$ T-helper cells and the induction of $FoxP3^+$ Treg. An important role for initial antigen presentation in the liver draining portal/celiac lymph nodes by professional as well as liver resident antigen-presenting cells (APCs) is indicated.

therapy of FVIII. Long-term tolerance to FVIII, which resisted subsequent re-challenge with FVIII protein, was observed and was attributed to TGF-β1-dependent conversion of FVIII-specific $CD4^+CD25^-$ conventional T cells into Treg (52, 53).

A different approach to utilizing Treg to suppress inhibitor formation is that of a cell therapy (**Figure 2**). For example, *ex vivo* expanded polyclonal $CD4^+CD25^+FoxP3^+$ Tregs are successfully used in hematopoietic stem cell transplants to prevent graft vs host disease are also evaluated in the treatment of autoimmune disease (54). Methods for the expansion of clinical-grade human Treg are well established and continuously further optimized (55). *Ex vivo* expanded Tregs highly up-regulate CTLA-4, enabling them to down-regulate the co-stimulatory signaling molecules CD80/CD86 upon interaction with DCs, thereby promoting tolerogenic antigen presentation (56, 57) (**Figure 2**). In the hemophilia A mouse, transplant of polyclonal Tregs, which had been *ex vivo* expanded with anti-CD3/-anti-CD28 beads and IL-2, suppressed inhibitor formation against FVIII protein therapy even after the transferred cells become undetectable (56). Adoptive transfer and *in vitro* studies revealed the ability of the expanded non-specific Treg to enhance induction of endogenous, antigen-specific Treg by facilitating conversion of conventional specific $CD4^+$ T cells to $CD4^+CD25^+FoxP3^+$ Treg (56) (**Figure 2**). This approach has the advantages of availability of clinical protocols and reagents and of not requiring genetic manipulation of patient cells. However, large cell numbers are likely required, and therefore, there is a risk of general immune suppression early after cell transplant (**Table 1**).

Hence, efforts are directed at generation of FVIII-specific Treg. This can be accomplished by redirecting antigen-specificity through TCR or chimeric antigen receptor (CAR) gene transfer to Treg (**Figure 2**). FVIII-specific Tregs generated by engineering Tregs to express a single human TCR (upon *ex vivo* retroviral gene transfer) have been found to suppress $CD4^+$ T-cell and B-cell responses and to be suppressive in hemophilia A mice expressing human HLA (58). Although these Tregs only recognized a single epitope, suppression of responses against the entire FVIII molecule occurred. Nonetheless, because of differences in HLA, translation of this strategy would require cloning of multiple TCRs. In contrast to transferring a TCR, the CAR approach is that it is not MHC restricted. Therefore, one construct could theoretically be used for all patients. CAR T cells are engineered by introducing antigen recognizing variable region (single-chain variable fragment) antibody domains, fused to primary and co-stimulatory signaling molecules. Antigen recognition and cell signaling by CAR expressing T lymphocytes are therefore independent of APCs and is not MHC restricted (59, 60). Successful trials of CAR T-cell immunotherapies for the treatment of leukemia have created possibilities of engineering FVIII-specific CAR Tregs, with antigen-specific suppression (61, 62). Experimentally generated FVIII-specific human CAR Tregs suppressed antibody formation *in vitro* and *in vivo* in hemophilia A mice (58, 63). CAR T cells typically recognize

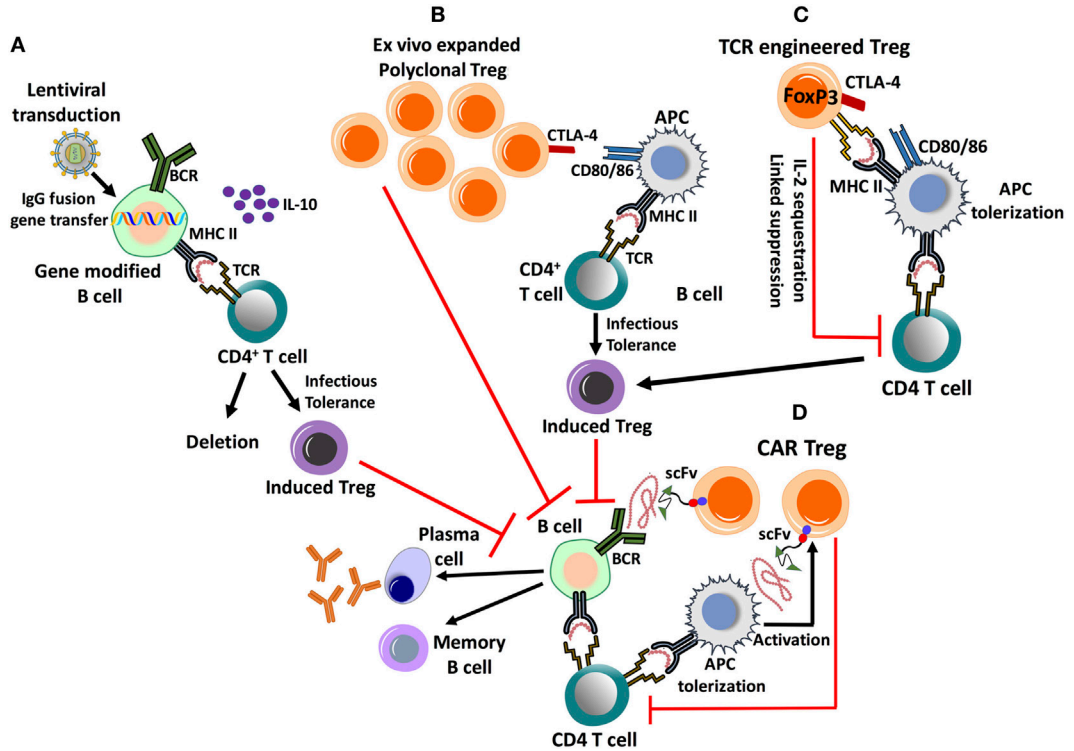

FIGURE 2 | Emerging cell therapy-based approaches to eliminate inhibitor formation. These proposed treatments are based on the *ex vivo* expansion/engineering of autologous lymphocytes, followed by adoptive transfer back into the patient. **(A)** Lentivirally transduced, gene modified B cells expressing an IgG fusion protein can induce tolerance by MHC II presentation of the clotting factor product, which results in the deletion of effector $CD4^+$ T cells and induction of $CD4^+CD25^+FoxP3^+$ regulatory T cell (Treg). Tolerance induction is dependent on IL-10 production. **(B)** *Ex vivo* expanded, polyclonal $CD4^+CD25^+FoxP3^+$ Treg highly up-regulate CTLA-4, promoting antigen-presenting cell (APC) tolerization by binding to co-stimulatory CD80/86 molecules. This facilitates the conversion of $CD4^+$ T helper cells into induced Treg by a process of infectious tolerance, subsequently leading to antigen-specific suppression. **(C)** Treg can be engineered to express a factor VIII-specific T-cell receptor (TCR), redirecting antigen recognition to a specific, MHC II-restricted epitope of the clotting factor. They can suppress $CD4^+$ T-cell and B-cell responses by multiple mechanisms. **(D)** Chimeric antigen receptor (CAR) Treg is engineered by introducing antigen recognizing single-chain variable fragment (scFv) antibody domains, fused to primary and co-stimulatory TCR signaling molecules. CAR Treg can recognize clotting factor bound to the surface of APC, leading to their activation and suppressive mechanisms, which include APC tolerization and $CD4^+$ T-cell inhibition. It is yet unknown whether CAR Treg can directly suppress antigen-specific B cells.

antigens of cell surfaces. The mechanism of suppression of responses to a soluble protein such as FVIII is, therefore, not entirely clear. For *in vitro* suppression, APCs were required, suggesting that cell surface association is needed (**Figure 2**). A related question is whether CAR Treg may be able to directly suppress B cells. Prior to translation of the approach, questions regarding the *in vivo* persistence, durability of suppression, and safety need to be addressed.

DIRECTLY TARGETING B CELLS FOR TOLERANCE

Upon activation, B cells differentiate into memory cells or antibody secreting cells, including plasma cells. However, B cells also play a role in antigen presentation (64). Interestingly, gene-modified primary B cells have the capacity to induce immune tolerance upon retroviral or lentiviral gene transfer (while TLR9 activation during plasmid gene transfer generates immunogenic B cells) (65–67). Skupsky et al. showed that the expression of IgG fusion proteins (IgG-A2 and IgG-C2 domains of FVIII) in primary B cells is a particularly powerful tool to induce tolerance (65). Gene-modified B cells route the expressed fusion protein through the endosomal compartment, resulting in MHC II presentation, deletion of effector T cells, and induction of $CD4^+CD25^+FoxP3^+$ Treg (**Figure 2**) (68). Adoptive transfer of a mixture of retrovirally transduced B cells, expressing IgG fusion of FVIII A2 or C2 domain, suppressed inhibitor formation in hemophilia A mice (69). Similarly, IgG-F9 gene transfer prevented and reversed inhibitor formation and anaphylaxis against FIX in mice with hemophilia B (FIX deficiency) (66). A major limitation of this approach had been a lack of suitable gene transfer vectors for human B cells. Recent development of lentiviral vectors (LV) targeted to human CD20 through inclusion of a single chain antibody fragment in the viral envelope protein has overcome this bottle neck (67). Primary B cells transduced with such a LV to express IgG-FIX prevented inhibitor formation in hemophilia B mice (67). However, clinical translation is still not straightforward because of relatively low titers of this vector.

TABLE 1 | Summary of main approaches currently being developed for tolerance induction to factor VIII (FVIII).

Approach	Mechanism	Advantages	Disadvantages
Hepatic gene transfer	Induction of FoxP3+ regulatory T cells (Tregs) and deletion of effector T cells	Combines treatment with immune tolerance induction; potential for inhibitor reversal; already in advanced clinical development as a therapy for adults	Requires gene transfer to pediatric patients; immune responses to viral vectors have been observed clinically
Co-administration of FVIII with rapamycin (potentially combined with cytokines)	*In vivo* induction of FoxP3+ Treg combined with deletion of effector T cells by inhibition of mTOR pathway	Lasting tolerance induction after transient regiment	Transient general immunosuppression
Ex vivo expansion of polyclonal Treg	Down-regulation of co-stimulatory molecules CD80/CD86, promoting tolerogenic antigen presentation and endogenous Treg induction	Clinical protocols already established	Transient immune suppressive effects/initial lack of antigen-specificity, large number of cells required
Ex vivo gene-modified Tregs	FVIII-specific suppression by FoxP3+ Treg with specificity redirected by T-cell receptor (TCR) or chimeric antigen receptor (CAR) gene transfer	Reduced cell numbers for therapy, antigen-specificity, no MHC restrictions for CAR approach	Genetic manipulation of patient cells required, MHC restriction for the TCR approach, durability, and costs unclear
Ex vivo gene modified B cells	Treg induction and effector T-cell deletion via MHC II presentation by transduced B cells	Highly effective in animal models	Use of integrating vectors required, large number of cells required, limitations to titers of current lentiviral vectors that transduced human B cells
B-cell depletion with rituximab (anti-CD20)	Depletion of CD20+ B cells	Reduces inhibitors in some patients that failed traditional immune tolerance induction, can potentially be combined with rapamycin to induce tolerance in such patients	Does not target plasma cells, inhibitors tend to relapse (although the outcome may be improved by combination with other drugs such as rapamycin)
Oral tolerance	Bioencapsulation and targeting of antigen to immune system of small intestine, induction of FoxP3+ and LAP+ Tregs	Could be considered prophylactically, antigen-specific tolerance without immunosuppression or genetic manipulation, low production cost	Repeat oral delivery appears required for lasting tolerance

Theoretically, protocols directed at elimination of memory B cells should benefit patients with persistent inhibitors, in particular those who fail to respond to traditional ITI. Hence use of the monoclonal antibody rituximab, which eliminated CD20+ cells in the human body through a variety of mechanisms, has been tested for this purpose (70). A recent Phase II safety study demonstrated that the anti-CD20 antibody rituximab showed a modest affect in reducing inhibitors and preventing anamnestic responses to repeated exposures to FVIII, albeit relapse of the inhibitor response remains a problem (71). B-cell depletion by itself may not be effective for tolerance induction, which likely requires tolerance induction in the T-cell compartment. Therefore, combination therapy with additional drugs may be needed. Interestingly, a recent study showed that combining anti-CD20-mediated B-cell deletion with rapamycin and FVIII antigen substantially improved reversal of inhibitor formation in hemophilia A mice (72). Such a protocol was superior to anti-CD20 or rapamycin alone or to polyclonal Treg therapy. B-cell depletion was also shown to enhance tolerance induction to FVIII in the context of hepatic gene transfer, when transgene expression was low and thus ineffective in Treg induction (73).

HEPATIC GENE THERAPY FOR TOLERANCE INDUCTION

In contrast to protein replacement therapy, gene therapy has the potential for a lasting cure of hemophilia. A first successful gene therapy for hemophilia B, utilizing *in vivo* gene transfer to the liver with an AAV vector, was documented in recent years (74). Currently, there are multiple clinical trials using liver-directed AAV vectors to treat hemophilia A and B (75). Some of these are achieving levels of FVIII or FIX activity at or near normal, and are thus expected to progress to Phase III trials (76). AAV vectors are comprised of a DNA genome (that is either single-stranded or modified to be self-complementary) packaged into a protein capsid. These vectors, derived from a small non-pathogenic parvovirus, lack viral coding sequences and effectively transfer genes *in vivo*. Viral capsids with a tropism for the liver are utilized in current trials, and the therapeutic gene is under transcriptional control of a hepatocyte-specific promoter. Given the limited packaging capacity of the vector (~5 kb), B domain-deleted FVIII (BDD-FVIII) is expressed. The B domain is dispensable for FVIII activity, and several recombinant FVIII products are BDD. The liver is an ideal target organ for gene therapy for hemophilia. FVIII and FIX are normally synthetized by liver sinusoidal endothelial cells (LSECs) and hepatocytes, respectively (77). Both cell types can efficiently secrete proteins into circulation.

From an immunological point of view, hepatic gene transfer has the major advantage that transgene expression in the hepatic environment can induce immune tolerance (78) (**Figure 1**). Given their hepatotropism, low innate immunity (resulting in limited tissue inflammation at the time of gene transfer), and the inefficiency in transducing professional APCs, AAV vectors

derived from several serotypes are ideal to achieve immune tolerance by hepatic gene transfer. Higher expression levels, as determined by vector serotype, dose, and the transgene expression cassette, favor tolerance induction (61, 79). High expression of the antigen enhances Treg induction and direct inhibition of memory B cells (80).

Constitutive exposure to FIX by hepatic gene transfer has been associated with $CD4^+$ and $CD8^+$ T-cell unresponsiveness and deletion by programmed cell death using both extrinsic and intrinsic mechanisms (79, 81, 82). In mice injected with AAV expressing the model antigen ovalbumin (AAV8-OVA), transgene-specific $CD8^+$ T cells transiently up-regulated negative checkpoint markers, e.g., the programmed death 1 receptor, leading to inefficient killing of transduced hepatocytes. Tolerance induction to FVIII or FIX has been shown to rely on the induction and enrichment of $CD4^+CD25^+FoxP3^+$ Tregs, which suppressed $CD8^+$ T cells and antibody formation in both mice and non-human primates (79, 83–85). Studies in Fas-deficient mice suggested that Treg induction and T-cell deletion were both required for robust tolerance induction (79, 84). Intrahepatic IL-10 expression further enforced suppression of $CD8^+$ T cells, without affecting antibody levels, while TGF-β expression was required for both Treg induction and to control transgene antibody formation in AAV-hFIX transduced hepatocytes (86). Engagement of the transmembrane protein GITR enhanced the proliferation and suppressive capacity of induced Tregs (84, 87, 88).

Antigen presentation in the tolerogenic liver environment by both professional APCs and liver resident cells is not fully understood but orchestrates a balance between immune regulation and immune surveillance (89). Administration of hepatotropic AAV8-OVA identified $CD11c^+$ DC and macrophages as APCs that are required for MHC II presentation of the transgene product, which primarily occurs in liver draining lymph nodes, such as the portal and celiac lymph nodes, although the liver itself may also contribute to Treg induction (**Figure 1**) (90). Liver-induced Treg rapidly disseminated through the circulation into multiple lymphoid organs, which resulted in systemic regulation of the response to the AAV gene product.

There have been concerns regarding the potential for cellular stress in the liver by over-expression of FVIII in hepatocytes. Improvements such as codon optimization of the F8 gene and deletion of the B domain have, however, resulted in only a mild activation of the unfolded protein response in mice, which did not impact liver pathology or FVIII immunogenicity (91, 92). Codon optimization of the F8 gene yielded higher hepatic expression levels that sustained therapeutic expression and improved tolerance induction (73). Translation of these studies in a large animal model for hemophilia A showed that AAV-mediated liver gene transfer of canine FVIII was not only effective in long-term sustained expression of FVIII but may also eradicate pre-existing inhibitory antibodies in two strains of hemophilia A dogs, with indications for improving outcomes in patients with established inhibitors (93–95).

The capacity of liver-directed gene transfer to induce immune tolerance to transgene products has also been demonstrated for LV. The large gene carrying capacity of LV makes them attractive candidates for hemophilia A gene therapy. Improved safety profiles have been reported with the development of hepatocyte-targeted, integrase-defective LV, which resulted in a sustained expression of FIX, tolerant to neutralizing antibody induction in hemophilia B mice, and without the risk of insertional mutagenesis (96). LV more efficiently transduce a variety of APCs, leading to innate immune responses, including TLR7 and TLR9 activation, and the induction of type I interferon and pro-inflammatory cytokines (97, 98). Ultimately, this immune activation drives $CD8^+$ T-cell and antibody responses against the transgene product. Transcriptional and post-translational engineering of the LV, using a combination of cell-specific promoters and miRNA target sequences to eliminate transgene expression in professional APCs (miR-142-3p), while restricting high levels of therapeutic expression to hepatocytes, has been shown to induce tolerance in both hemophilia A and B models and correction of disease phenotype (99–102).

A recent study has shown that directing LV-mediated FVIII gene expression to LSECs (which are the physiological source of FVIII synthesis) by using an endothelial cell-specific promoter, similarly resulted in stable and therapeutic levels of FVIII in mice (103, 104). Interestingly, using a CD11b myeloid cell-specific promoter and a target sequence for miR-126, which is highly expressed in endothelial and pDCs, resulted in the prevention of inhibitory antibodies to FVIII. Even after subsequent challenge of these mice with FVIII in adjuvant, they remained tolerant for up to 24 weeks. Therefore, an important contribution of gene transfer to pDCs in driving an immune to the FVIII antigen in LV gene therapy was proposed.

ORAL TOLERANCE INDUCTION USING TRANSGENIC CROP PLANTS

The immune system of the small intestine has evolved to promote tolerance to food antigens (105, 106). This phenomenon can be exploited in tolerance induction through oral antigen delivery. This concept has multiple advantages, since no immune suppressive drugs, genetic manipulation of host cells, or expensive cell therapies are required. Oral tolerance has been studied for more than half a century and is defined as a systemic immunological unresponsiveness or hyporesponsiveness to an orally administered antigen. Several recent successes in prevention of food allergies illustrate relevance for human treatment (107, 108). In experimental models of autoimmune diseases, orally administered antigens suppressed autoimmunity in animal models of experimental autoimmune encephalomyelitis, diabetes, and rheumatoid arthritis (109–111).

The mammalian digestive system has a rich and complex immune network that has evolved to maintain a delicate balance between tolerance and immunoreactivity (112, 113). The gut-associated lymphoid tissue consists of intestinal epithelial lymphocytes, concentrated within the intestinal epithelial barrier, Peyer's patches, and mesenteric lymph nodes (MLNs) (114, 115). The majority of the incoming food proteins get digested and degraded in the stomach and upper intestine. Proteins that escape degradation pass through the gut epithelial barrier and are

sampled by APCs. Antigen loaded APCs subsequently migrate to the MLNs (116, 117), where APCs activate and prime naïve antigen-specific T lymphocytes (118). Lillicrap and colleagues initially tested this mucosal tolerance concept for treatment of hemophilia A. Mice exposed to the immunogenic C2 domain of FVIII (FVIII-C2) via oral or nasal route developed partial tolerance to systemic challenges with FVIII-C2 and full-length FVIII. This tolerance persisted after adoptive transfer of $CD4^+$ splenocytes from FVIII-KO mice that received intranasal antigen administration (119).

However, for the concept to go forward, one had to develop a technology for cost effective production of the FVIII antigen, protection from degradation in the stomach, and effective delivery to the gut immune system. Answers to these challenges came with advancements in plant genetics, resulting in high levels of expression of human therapeutic proteins in the chloroplast (120, 121) of crop plants for the production of edible biopharmaceuticals (122). Initial experiments conducted in hemophilia B mice using frozen and ground tobacco leaves demonstrated robust suppression of inhibitor formation and of fatal anaphylactic reactions against intravenous FIX (123). In subsequent studies in hemophilia A mice, using a mixture of frozen tobacco leaves expressing either C2 domain or the heavy chain of human BDD-FVIII, effective suppression of inhibitor formation was documented in two different strains of hemophilia A mice (124). In both studies, the bioencapsulated antigens were given by oral gavage twice per week, starting 1 month prior to initiation of traditional replacement therapy. This method could also reverse FIX inhibitors and desensitize from the allergic reactions to FIX in hemophilia B mice, as well as accelerate the decline of pre-existing FVIII inhibitors in hemophilia A mice (124, 125).

Toward an oral tolerance protocol that is feasible in humans, transgenic lettuce plants were developed. This became feasible after identification of lettuce chloroplast-specific posttranscriptional elements that ensure high expression (126, 127). Furthermore, chloroplast genomics tools were developed for the identification of ribosomal stall sites and optimization of codon usage (128). In addition, growth of the transplastomic lettuce in a hydroponic system suitable for GMP production was developed, as well as a lyophilization process to generate leaf material for stable long-term storage at ambient temperature. When tested in hemophilia B mice, lyophilized lettuce containing human FIX was effective in tolerance induction over a wide range of antigen doses (127). To prove that the method is not limited to rodent models, a study in hemophilia B dogs was performed. These animals are similar in size to pediatric patients and reproducibly form antibodies against human FIX after repeated intravenous delivery. Three of the four dogs that received the oral tolerance regimen at an antigen dose of 0.3 mg/kg showed robust suppression in IgG and IgE formation against human FIX, correlating with a lack of inhibitor formation, lack of anaphylactic reactions, and restoration of blood clotting times after each of 8 weekly FIX injections (129). Extensive serum chemistry, hematological, and general health evaluations showed absence of toxicity even after several months of oral delivery.

The mechanism of plant induced oral tolerance is complex. The plant cell wall provides bioencapsulation for the antigens, which are released in the small intestine through degradation by enzymes produced by intestinal microbes. For efficient delivery across the gut epithelium, FVIII and FIX antigens are expressed in chloroplasts as N-terminal fusions to cholera toxin B (CTB) subunit. CTB is an effective transmucosal carrier that, in the form of a pentamer, binds to the GM1 receptor on the surface of epithelial cells (and other cell types, including DCs), resulting in uptake and translocation through transcytosis/retrograde trafficking (130). Inclusion of a furin cleavage sites assures release of FVIII or FIX sequence from CTB. Immunohistochemistry showed delivery to DCs in the lamina propria and in Peyer's patches (125). These include $CD103^+$ DC (**Figure 1**). Upon intravenous challenge with antigen, increases in the frequencies of $CD103^+$ DC and pDCs are observed, especially in MLNs. $CD103^+$ DC are critical APCs in oral tolerance induction, since they transport antigen to the MLN, where they effectively induce Treg. The plant cell-based oral tolerance protocol induces two subsets of Treg that systemically suppress antibody formation against FVIII or FIX, namely $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^-FoxP3^-LAP^+$ Treg (124, 125, 127) (**Figure 1**). The latter are most robustly induced and produce IL-10 and TGF-β cytokines (125). LAP^+ Tregs express latency-associated peptide (LAP) on the cell surface and suppress through a TGF-β dependent mechanism (131). Future work may show whether $FoxP3^+$ and LAP^+ Tregs have redundant or synergistic roles in oral tolerance induction to coagulation factors. Interestingly, there was also evidence for induction of type 1 Tregs in the lamina propria. These may locally contribute to tolerance induction through IL-10 expression. In fact, the oral tolerance mechanism was found to be IL-10 dependent, consistent with the notion that IL-10 is a key component of immune tolerance on mucosal interphases (125).

OTHER APPROACHES

Maternal antigen transfer may offer hope for many genetic disorders that are diagnosed *in utero*. The advantages of this method are as follows: immaturity of immune system, absence of pre-existing antibodies, and early, antigen-specific tolerance induction (132, 133). A recent study in hemophilia A mice found that intravenous administration of Fc fusions of FVIII A2 and C2 domains resulted in effective antigen transfer into the developing fetal immune system via the neonatal Fc receptor (132). Moreover, the offspring of injected females showed robust tolerance to repeated challenges with FVIII when compared with offspring of non-treated mothers. A window for tolerance induction during gestation was identified, resulting in development of thymic-derived and peripherally induced antigen-specific Treg. A potential limitation for this approach is the large antigen dose that may be required for effective transfer to the fetus. Interestingly, Fc-conjugated FVIII is in advanced clinical development as a method to increase the half-life of FVIII during replacement therapy (12, 134). Building on the tolerogenic properties of Fc sequences, these molecules may be superior antigens for ITI in general (17).

In other studies, neonatal AAV gene transfer to hemophilia A mice directed sustained therapeutic FVIII expression (~5% of normal) and immunological unresponsiveness, with no antibodies being detected against AAV or FVIII. Mice also remained tolerant to a subsequent FVIII challenge in adjuvant, performed 8 weeks after gene transfer (135). Alternatively, activated platelets can serve as a vehicle to deliver FVIII to the site of vascular injury in patients with inhibitors. Transplantation of modified hematopoetic stem cells (HSCs) with FVIII under megakaryocyte-specific promoter restored hemostasis in hemophilia A mice with inhibitors (136). Here, FVIII is stored in α-granules, which protects FVIII from elimination by inhibitors, which would occur for FVIII that circulates in plasma. Activated platelets release FVIII containing α-granules at the site of vascular injury, thereby restoring hemostasis. Similarly, LV-modified autologous canine megakaryocytes (precursors of platelets) expressing FVIII in α-granules prevented bleeding episodes in hemophilia A dogs (137). These large animal studies further support that platelet-derived FVIII may potentially benefit hemophilia patients with inhibitors. This approach combines autologous HSC gene transfer with bone marrow conditioning and has also been shown to tolerize the transplant recipient animals to FVIII (138).

In conclusion, a large number of diverse innovative approaches to induce immune tolerance in the treatment of hemophilia A and thus prevent and/or reverse inhibitor formation to FVIII are currently in pre-clinical development (**Table 1**). Mechanistically, these primarily aim at tipping the balance of the immune response to Treg induction. Each approach has conceptual advantages and disadvantages, which have to be factored into decisions about translation studies in humans (**Table 1**). Since inhibitors form in young boys with hemophilia, an acceptable level of immune suppression or genetic manipulation would have to be determined for some of these approaches. Nonetheless, new superior technologies for antigen-specific ITI hold much promise to finally reduce inhibitor formation in the treatment of hemophilia A patients.

AUTHOR CONTRIBUTIONS

RH wrote parts of the article. He supervised generation of the article and edited it. AS and MB wrote parts of the article and generated the figures.

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Endothelial Cell Amplification of Regulatory T Cells Is Differentially Modified by Immunosuppressors and Intravenous Immunoglobulin

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Immunosuppressive treatment is a prerequisite for both organ transplantation and tolerance of the allograft. However, long-term immunosuppression has been associated with a higher incidence of malignancies and infections. Immunosuppressors mainly target circulating immune cells and little is known of their "off-target" effects, such as their impact on endothelial cells (ECs). In chronic antibody-mediated rejection (AMR), the allograft endothelium is a target of damage, histologically detected as transplant glomerulopathy, and which correlates with poor graft survival. Under inflammatory conditions, EC expression of HLA class II antigens can lead to $CD4^+$-T lymphocyte alloactivation and selective expansion of pro-inflammatory Th17 and pro-tolerance Treg subsets. This response can be modified and preactivation of the EC by HLA-DR antibody binding promoted a proinflammatory Th17 response. However, whether or not immunosuppressors alter EC immunogenicity has not been examined. In alloimmunized patients with AMR, cyclosporine A (CsA) and mycophenolic acid (MPA) are often combined with intravenous immunoglobulins (IVIgs). This study reports changes in the microvascular EC phenotype and function after treatment with CsA, MPA, or IVIg. Both CsA and MPA decreased HLA-DR and increased CD54 expression, whereas IVIg increased HLA-DR expression. Interleukin 6 secretion was reduced by all three immunomodulators. Preincubation of ECs with CsA or MPA limited, while IVIg amplified, Treg expansion. Because CsA, MPA, and IVIg are known for their ability to act upon leukocytes, we confirmed that ECs maintained their immunoregulatory role when allogeneic leukocytes were pretreated with CsA, MPA, or IVIg. The results reveal that individual immunosuppressors, used in the induction and maintenance of renal allograft tolerance, had direct and distinct effects on ECs. Results of experiments associating IVIg with either CsA or MPA underlined the differences observed using individual immunosuppressors. Paradoxically, CsA or MPA may increase EC mediated inflammatory responses and long-term exposure may contribute to limitation of allograft tolerance. In contrast, IVIg interaction with the endothelium may mediate some of its immunosuppressive effects through promotion of Treg expansion, contributing to the maintenance of allograft tolerance.

Keywords: HLA, Th17, cyclosporine A, mycophenolic acid, endothelium, organ transplantation, interleukin 6, CD54

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INTRODUCTION

Organ transplantation is the treatment of choice for end stage renal disease, however establishing conditions for optimal and continued graft survival is a major challenge. Immunosuppressive therapy is essential for establishing allograft tolerance, which protects the graft from damage and decreases the incidence of rejection.

The maintenance of immunosuppression is key to the prevention of acute and chronic rejections. Major improvements in graft survival resulted from the introduction of drugs (such as cyclosporine A (CsA), tacrolimus, mycophenolate mofetil and sirolimus) and antibodies (such as Basiliximab). However, chronic use of immunosuppressors is also associated with unwanted side effects, including endothelial dysfunction, an increased risk of malignancy and opportunistic infection. Long-term immunosuppressive regimens often combine a calcineurin inhibitor such as CsA and an inhibitor of nucleotide synthesis such as mycophenolic acid (MPA).

The prototypic calcineurin inhibitor, CsA is a lypophilic cyclic undecapeptide that prevents T lymphocyte proliferation by inhibiting the transcription of required cytokine genes (1) and thereby restricts T lymphocyte expansion in response to alloantigen presentation. CsA inhibits calcineurin phosphatase and so prevents the nuclear translocation of NF-AT (2) and other pathways of CsA activity include activation of JNK and of p38 MAP kinases (3). However, CsA administration has been implicated in the development of vascular complications, including hypertension and thrombotic microangiopathies (4). Furthermore, CsA treatment has been reported to induce oxidative stress and apoptosis in endothelial cells (ECs) and has been associated with enhanced EC damage and dysfunction (5–7).

Mycophenolate mofetil, the prodrug of MPA, is a reversible inhibitor of inosine 5′-monophosphate dehydrogenase (IMPDH). IMPDH is required for *de novo* purine biosynthesis and is responsible for the formation of guanine nucleotides from IMP to xanthosine 5′-monophosphate (8). Inhibition of IMPDH depletes the guanine nucleotide pool, decreases DNA synthesis and thereby reduces lymphocyte proliferation (8, 9). MPA also affects differentiation and maturation of professional antigen presenting cells including dendritic cells and B lymphocytes. MPA treatment reduces dendritic cell activation of T lymphocytes *via* the indirect pathway of antigen presentation and inhibits antibody production by lowering the number of antibody-secreting B lineage cells; this occurs without altering the expression of HLA molecules on B lymphocytes (10). Lastly, IMPDH is involved in the synthesis of membrane glycoproteins and inhibition of IMPDH alters the expression of adhesion molecules.

Although not an immunosuppressor, polyclonal intravenous immunoglobulins (IVIgs) are used to treat allosensitized transplant patients and particularly those with high levels of circulating donor specific antibodies. In contrast to the limited experience with polyclonal IVIg in transplantation, IVIg have been widely used in the treatment of autoimmune diseases (11, 12) and the efficiency of IVIg in various pathologies, including antibody-mediated rejection (AMR), has been recently reviewed (13). IVIgs are pooled preparations of immunoglobulins isolated from plasma obtained from more than 1,000 healthy donors per batch. IVIg is frequently used in the treatment of various autoimmune and immune-mediated inflammatory conditions (e.g., idiopathic thrombocytopenic purura, Kawasaki disease, dermatomyositis, systemic lupus erythematosus, and Sjogren's syndrome). IVIg has been successfully used for the densensitization of renal and heart transplant patients with alloantibodies (14–16). Multiple and mutually non-exclusive mechanisms have been proposed to explain the benefits of IVIg including: effects on B and T lymphocytes, macrophages, dendritic cells, and natural killer cells (17–23). IVIg treatment has been reported to regulate activation and induction of Treg, to enhance Treg function by increased expression of the transcription factor FoxP3, by production of the cytokines transforming growth factor β and interleukin 10 (IL-10), and by cytotoxic T lymphocyte antigen expression (24–27).

The activity of immunosuppressors has been extensively studied in circulating immune cells, particularly T lymphocytes and professional antigen presenting cells (such as dendritic cells), because of the crucial role of these cells in the maintenance of tolerance. However, studies have largely neglected to assess the impact of immunosuppressors on EC function. Intragraft microvascular ECs form the barrier between the allograft and the recipient, and thus are in direct contact with the host immune system and the administered immunosuppressors. In the context of chronic AMR, the importance of ECs in allograft tolerance is underlined by the association between endothelial lesions and a heightened level of expression of EC activation-associated transcripts. Microvascular inflammation is also a histological indicator of AMR (28, 29).

Allograft microvascular ECs express HLA class II antigens in the steady-state and expression is highly increased under inflammatory conditions (30, 31), allowing $CD4^+$-T lymphocyte activation by these cells (32). It has been reported that incubation of ECs with CsA reduced MHC class II-mediated presentation of antigen by altering intracellular antigen processing (33, 34). Regarding MPA, reduced dendritic cell expression of CD80, CD83, CD86, and CD205 has been reported, whereas HLA-DR expression was unaltered (10). In contrast, MPA treatment was associated with a dose-dependent decrease in HLA-DR expression in B lymphocytes and the same trend was observed in T cells (9).

Our previous studies revealed that HLA-DR expressing ECs regulate the allogeneic $CD4^+$-T lymphocyte response, selectively and simultaneously promoting an IL-6/STAT-3-dependent pro-inflammatory Th17 response and a contact and CD54-dependent expansion of functionally suppressive $CD4^+CD45RA^-HLA\text{-}DR^+FoxP3^{bright}$ Treg (35). Studies in organ transplantation have conferred conflicting roles on Treg and Th17 (2) and several reports suggest that the intragraft localization of Treg is associated with decreased rejection and improved graft survival, while Th17 have been associated with promoting rejection in renal transplantation (36, 37). In humans, correlations between the proportion of Tregs within allografts and graft survival have been observed (38–40) leading to several ongoing trials of Treg infusion following organ transplantation (41). We have reported that EC allogenicity can be regulated by environmental factors. For example, inhibition of IL-6 binding promoted the Treg response

whereas binding of alloantibody directed against endothelial HLA class II antigens selectively increased IL-6 secretion and the Th17 response (35, 42).

Regarding adhesion molecule expression by ECs, studies of the activity of MPA in macrovascular human umbilical vein or aortic ECs have shown conflicting results. MPA increased VCAM-1 (43), decreased ICAM-1 and VCAM-1 (44, 45), or had no effect on ICAM-1 expression (43) in TNF-α-stimulated ECs. The increased expression of VCAM-1 appeared to result from the increased stability of mRNAs (43). Olejarz et al. showed that the reduction of ICAM-1 was due to inhibition of a ROS-dependent MAP kinase, which regulates NF-κB activation and nuclear translocation (45).

Despite the many studies of immunosuppressor activity in leukocytes, their effect on endothelial allogenicity has not been addressed. We have investigated the impact of CsA, MPA, and IVIg on ECs in an inflammatory environment. The immunosuppressors MPA and CsA induced phenotypic changes, some of which were associated with altered mRNA expression. Functional studies revealed altered immunogenicity of ECs resulting in a predominantly proinflammatory $CD4^+$-T cell response. In contrast, IVIg induced mRNA and phenotypic changes which were associated with a higher Treg response. Importantly immunosuppressor-promoted proinflammatory, and IVIg-promoted regulatory responses were maintained after exposure of allogeneic leukocytes to immunosuppressors suggesting that ECs could be active in systemic immunosuppression. Moreover, IVIg retained its activity on ECs when used in combination with MPA or CsA. These data contribute to understanding the effects of immunosuppressors on the endothelium and may also expose a hitherto unknown regulatory pathway initiated by IVIg interaction with ECs.

MATERIALS AND METHODS

Cells Lines and Culture Reagents

The HMEC-1 cell line was cultured as previously described and used between passages 8 and 18 (26). Coculture experiments were carried out with ECs and non-HLA matched PBMCs, as reported (28). PBMCs were isolated from healthy donor blood samples (obtained in accordance with institutional regulations from the Etablissement Français du Sang, Paris, France) by Ficoll density gradient separation (Eurobio, Les Ulis, France).

ECs Pretreatment with Immunosuppressors

Endothelial cells were cultured with interferon γ (IFN-γ) (200 IU/ml for 3 days; R&D Systems, Minneapolis, MN, USA) in tissue culture flasks and incubated, where indicated, with immunosuppressors: MPA (Sigma-Aldrich), CsA (Sandimmun®, Novartis), or with IVIg (Privigen®, CSL Behring) at the indicated concentrations. Parallel cultures were carried out with the relevant diluent before phenotypic, qPCR and functional assays. Methanol and ethanol were used for suspension of MPA and of CsA, respectively. Polyclonal IVIg was diluted in tissue culture medium. In certain experiments cells were pretreated with combinations of either CsA and IVIg or MPA and IVIg at the indicated concentrations.

EC Apoptosis Assay

Cells were seeded at a density of 20,000 cells/cm^2 and treated with IFN-γ and immunosuppressors as described above. After 3 days of treatment, apoptosis was calculated by flow cytometry using PE Annexin V apoptosis detection kit from BD Pharmingen (BD Biosciences) according to the manufacturer's instructions.

Real-time Polymerase Chain Reaction Analysis

CD54, HLA-DR, IL-6 and Glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNAs were assayed using a fluorescence-based real-time PCR. After 3 days of treatment with immunomodulators, total RNA was isolated from ECs using the TRI Reagent (Ambion, Applied Biosystems, Thermo Fischer Scientific) protocol. RNA was quantified using a spectrophotometer (ND-1000; Nanodrop), and converted to cDNA (1 µg RNA/reaction) by reverse transcription (RT) using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies). Real-time PCR was performed with ViiA 7 Real-Time PCR System (Applied Biosystems, Thermo Fischer Scientific) and TaqMan gene Expression Assay (Applied Biosystems, Thermo Fischer Scientific). The primers and probe sets used for this study were: IL-6 (Hs00174131_m1), CD54 (Hs00164932_m1), HLA-DR (Hs00219575_m1), and GAPDH (Hs027558991_g1). Threshold cycles (Ct) were determined as the mean of duplicate determinations. The differences in relative abundances of mRNA were calculated as $2^{-\Delta(\Delta Ct)}$.

Allostimulation Assays

After incubation of ECs with IFN-γ in the presence or absence of individual or combinations of immunosuppressors, they were washed and incubated in fresh medium overnight. Cells were then irradiated (20 Gy) and cocultured with PBMCs at a ratio 1:1 for 7 days as described (42). We have previously confirmed that the irradiation step did not prevent cytokine secretion within the following 3 days (42). In indicated experiments, PBMCs from healthy donors were incubated in the presence of immunosuppressors for 24 h before coculture. The supernatants of cocultures were collected after 72 h for cytokine measurement. At the end of the coculture, PBMCs were stimulated by phorbol-12-myristate-13-acetate 50 ng/ml, and ionomycin 1 µM (Cell Signaling Technology) in the presence of GolgiStop (BD Biosciences) for 4 h before labeling cells to detect T lymphocytes expressing intracellular IL-17 ($CD3^+CD8^-$IL-17^+) or IFN-γ ($CD3^+CD8^-$IFN-γ^+) by flow cytometry. Carboxyfluorescein succinimidyl ester-labeled PBMCs were used to determine proliferation of Treg ($CD4^+$ $CD45RA^-FoxP3^{bright}$) and Tmem ($CD4^+CD45RA^-FoxP3^{low}$) subpopulations.

Antibodies and Flow Cytometry

For phenotypic analysis of $CD4^+$-T, the following antibodies were used: CD4 PE (Clone RPA-T4), IFN-γ FITC (Clone B27), HLA-DR allophycocyanin (APC) (clone G46-6) (BD

Pharmingen; BD Biosciences), CD3 PerCP (clone SK7) (Becton Dickinson, Franklin Lakes, NJ, USA); CD4 PB (Clone RPA-T4), CD8 PB (Clone RPA-T8), CD45RA PE/Cy7 (clone H100), CD25 PE (clone M-A251), CD127 PerCP/Cy5.5 (clone A019D5), CD185 APC/Cy7 (clone J252D4) (Biolegend); and CD54 Pacific Blue (clone HCD54), IL-17 efluor660 (eBioscience). Intracellular staining of FoxP3 was carried out with the anti-Human Foxp3 Staining Set APC (clone 236A/E7) (eBioscience). Flow cytometry was carried out on a FACS Canto II (BD Biosciences).

IL-6 Detection by Enzyme-linked Immunosorbent Assay

Assessment of IL-6 was carried out in supernatants of EC cultures or cocultures with PBMC, using an enzyme-linked immunosorbent assay detection kit from BD Biosciences, and according to the manufacturer's protocol. All samples were assayed in duplicate.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The statistical significance of the data was determined using the indicated tests ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$).

RESULTS

Immunosuppressive Treatments Modify the Phenotype of Microvascular ECs

Cell-surface expression of HLA-DR and CD54 molecules is required for amplification of Tregs, and expansion of the Th17 subset is dependent on endothelial secretion of IL-6 (35, 42). We therefore assessed the phenotypes of EC after treatment with immunosuppressors.

Following incubation with MPA, an increased proportion of CD54 expressing cells and of CD54 expression was observed over a range of concentrations (**Figure 1A**; Figures S1and S2 in Supplementary Material). Similarly to MPA, pretreatment with CsA increased the percentage of CD54 expressing ECs and their level of expression at most concentrations tested (**Figure 1B**; Figures S1 and S2 in Supplementary Material). In common with the immunosuppressors, IVIg treatment also led to an increase in the proportion of CD54 expressing ECs and in the level of expression (at concentrations ranging from 5 to 20 mg/ml) (**Figure 1C**; Figures S1 and S2 in Supplementary Material).

Expression of HLA-DR was reduced by MPA both in terms of the percentage of cells and the intensity of expression at all concentrations tested (**Figure 1D**; Figures S1 and S2 in Supplementary Material). While CsA also decreased HLA-DR expression, this was only observed at the highest concentrations tested (**Figure 1E**; Figure S2 in Supplementary Material). The effect of MPA on HLA-DR expression was much more marked than that of CsA and even the lowest concentration tested (3 μg/ml) reduced HLA-DR expression to 57.1% of the control level ($p < 0.05$). Finally, if MPA was added after stimulation of ECs with IFN-γ, the decrease of HLA-DR required an extended exposure to MPA (data not shown). In a previous study, the reduction of HLA-DR by MPA resulted from inhibition of guanosine synthesis, leading to an inability to transcribe mRNA and to synthesize new proteins (10).

In contrast to MPA and CsA, EC incubation with IVIg resulted in a modest but significant dose-dependent increase in the proportion of HLA-DR expressing ECs (**Figure 1F**; Figure S2 in Supplementary Material).

Our previous study of the signaling required for EC polarization of Th17 cells identified the role of IL-6. Both MPA and IVIg significantly reduced EC secretion of IL-6 in a dose-dependent manner (**Figures 1G,I**). The reduction of IL-6 was approximately 50% after cell incubation with the lowest concentration of MPA tested.

In contrast to MPA and IVIg, incubation with CsA led to a decrease in IL-6 secretion in the presence of 2.5 μg/ml but an increase at higher concentrations (10−20 μg/ml). The increased detection of IL-6 at high levels of CsA may be due to cell stress resulting in the release of stored IL-6. This is likely to be the case as detection of apoptosis after treatment of ECs with CsA, MPA, or IVIg revealed substantial apoptosis only after incubation with a high concentration of CsA (Figure S3 in Supplementary Material).

We next determined whether the changes in protein expression were related to modifications of mRNA expression. While MPA did not alter mRNA expression of CD54 (**Figure 2A**), both CsA and IVIg increased expression and this was particularly marked at high concentrations (**Figures 2B,C**). In contrast, mRNA levels of HLA-DR were strongly reduced by MPA (**Figure 2D**) [in agreement with the results of a previous study of MPA in human dendritic cells and B lymphocytes (10)]. However, HLA-DR mRNA levels were strongly increased by IVIg and not significantly altered by CsA (**Figures 2E,F**). When IL-6 expression was assessed, both MPA and IVIg reduced mRNA expression in agreement with the protein levels (**Figures 1G,I** and **2G,I**) whereas CsA decreased mRNA expression at the highest concentration tested (**Figure 2H**). It is possible that 10μg/ml CsA activates cell stress or death (Figure S3 in Supplementary Material) resulting in inhibition of IL-6 mRNA production and simultaneous release of IL-6 stores.

The increase in CD54 protein therefore corresponds with the increase in mRNA, this is also the case for the reduced HLA-DR expression observed with MPA and the increase observed with IVIg in addition to the decrease in IL-6 induced by either MPA or IVIg. However, modifications of mRNA expression by CsA corresponded less well to the changes in protein expression. Together these data indicate that the three immunomodulators differentially alter the protein and mRNA expression of CD54, HLA-DR, and IL-6 by ECs.

MPA, CsA, and IVIg Modify IL-6 Secretion in Cocultures of EC and PBMC

Because the immunosuppressors and IVIg directly influenced IL-6 secretion by EC we next examined whether EC treatment with immunomodulators modified IL-6 production in EC–PBMC cocultures, since it is this context that Th17 polarization by EC is observed. As shown in **Figure 3**, MPA treatment reduced IL-6 in cocultures by approximately 50% at the highest concentration

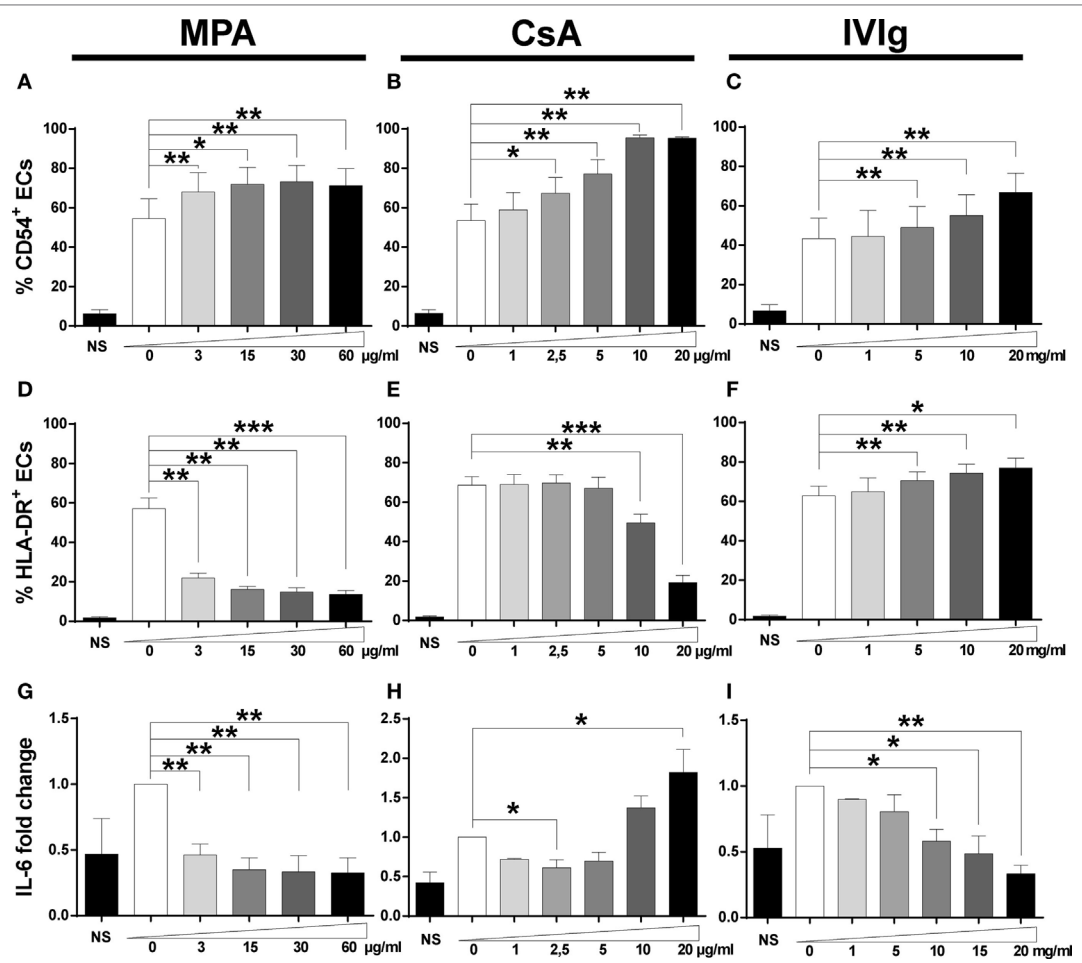

FIGURE 1 | Treatment with mycophenolic acid (MPA), cyclosporine A (CsA), or intravenous immunoglobulin (IVIg) modifies endothelial cell (EC) phenotype. The phenotype of ECs was studied after 3 days of incubation with interferon γ and MPA, CsA, or IVIg at the indicated doses. NS represents EC incubated in the absence of IFN-γ, immunosuppressors, or IVIg. Control values for ECs incubated with the vehicle solutions (methanol, ethanol, or medium for suspension of MPA, CsA, or IVIg, respectively) are represented as 0. The percentage of CD54 expressing cells is shown after treatment of ECs with MPA [**(A)**, $n = 5$], CsA [**(B)**, $n = 5$], or IVIg [**(C)**, $n = 6$]. [**(D)**, $n = 5$], [**(E)**, $n = 5$], and [**(F)**, $n = 6$] show the percentage of HLA-DR expressing cells after treatment of ECs with MPA, CsA, and IVIg. [**(G)**, $n = 5$], [**(H)**, $n = 5$], and [**(I)**, $n = 3$] show the effect on interleukin (IL)-6 secretion after treatment of ECs with MPA, CsA, and IVIg, respectively. The IL-6 secretion by ECs is expressed as fold change of the level produced by cells incubated with vehicle alone. The mean ± SEM values (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, paired *t*-test) are shown.

tested (**Figure 3A**). Concerning CsA, a dose dependent reduction of IL-6 secretion was also noted (**Figure 3B**). In contrast with the significant and dose-dependent reduction in IL-6 observed when EC alone were pretreated with IVIg (**Figure 3C**), IL-6 secretion was only slightly decreased in the setting of a coculture of IVIg pretreated EC and PBMC.

Immunosuppressive Treatments Do Not Alter the Differentiation of the Proinflammatory Th17 Subset by ECs

Th17 cells have been both directly and indirectly implicated in allograft rejection (37, 46). Increased Th17 differentiation by HLA-DR expressing ECs was related to proliferation of IL-17$^+$ memory T cells (Tmem) because inhibition of STAT-3 activation or of the IL-6 receptor decreased Th17 in association with decreased Tmem proliferation (35). We determined whether Tmem, or expansion of Th17 or Th1 subpopulations (Gating strategy shown in Figure S5 in Supplementary Material) are compromised by incubation of ECs with CsA, MCA or IVIg.

When the proportion and proliferation of CD4$^+$CD45 RA^{neg}FoxP3^{low} Tmem was determined in cocultures of PBMC with ECs pretreated with MPA, CsA, or IVIg, the only difference observed was the strongly reduced proliferation of Tmem induced by MPA (Figure S4B in Supplementary Material, $p < 0.05$ compared with non-treated control). EC treatment with either CsA or IVIg did not alter the proportion or the proliferation of Tmem.

Incubation with MPA, CsA, or IVIg before coculture with PBMC did not alter Th17 expansion (**Figures 3D–F**). However,

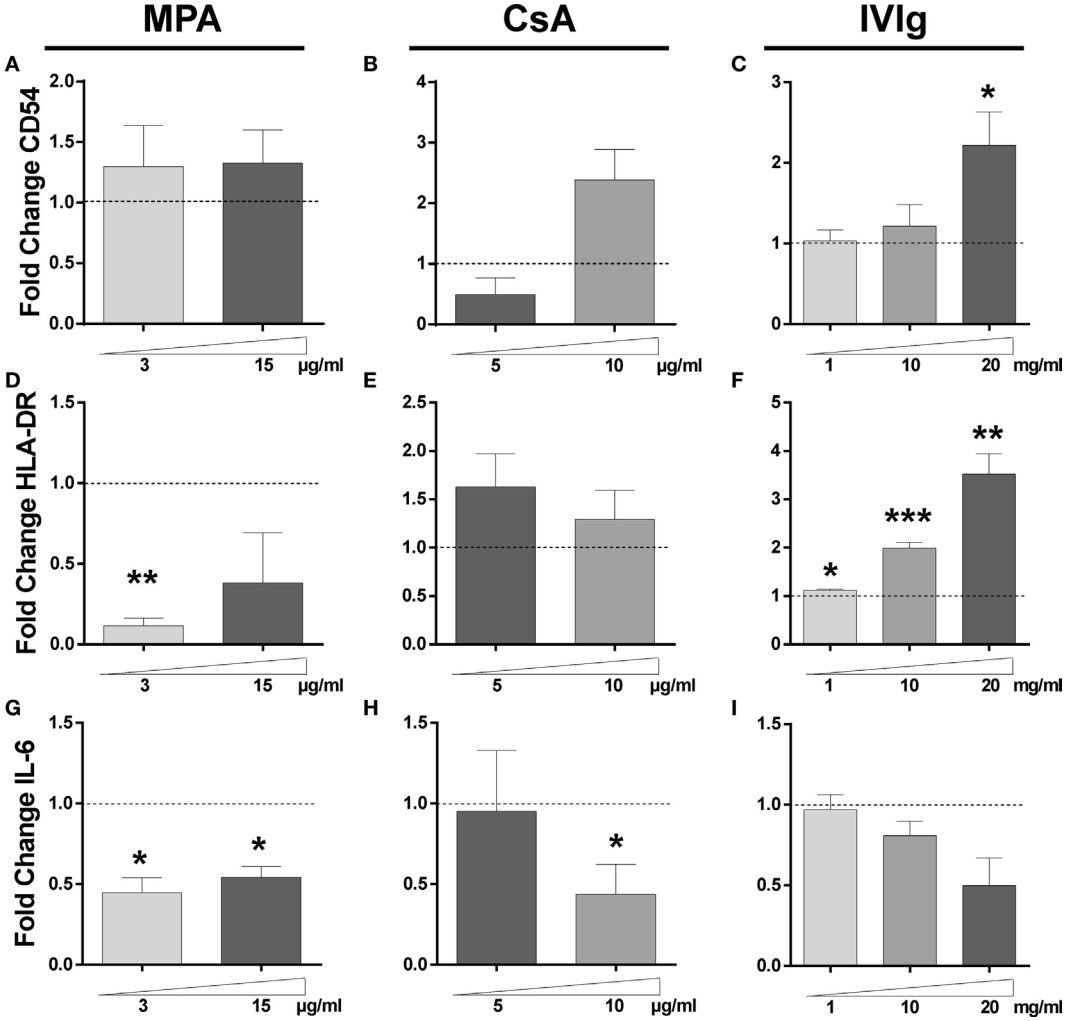

FIGURE 2 | Impact of Immunomodulators on endothelial cells (ECs) gene expression. The transcription of CD54, HLA-DR, and interleukin (IL)-6 genes in ECs was examined by qRT-PCR after 3 days of incubation with interferon γ and mycophenolic acid (MPA), cyclosporine A (CsA), or intravenous immunoglobulin (IVIg) at the indicated doses. Control values for ECs incubated with the vehicle solutions. The results are expressed as fold change of GAPDH gene expression. The relative transcription level of CD54 is shown after treatment of ECs with MPA [**(A)**, $n = 3$], CsA [**(B)**, $n = 4$], or IVIg [**(C)**, $n = 5$]. [**(D)**, $n = 3$], [**(E)**, $n = 4$], and [**(F)**, $n = 5$] show the transcription levels of HLA-DR gene after treatment of ECs with MPA, CsA, and IVIg. [**(G)**, $n = 3$], [**(H)**, $n = 4$], and [**(I)**, $n = 4$] show the effect on IL-6 transcription after treatment of ECs with MPA, CsA, and IVIg, respectively. For all graphs, the mean ± SEM are indicated (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, paired *t*-test).

there was a modest, but significant, increase in the differentiation of proinflammatory $CD3^+CD8^{neg}IFN\text{-}\gamma^+$ Th1 cells after preincubation of EC with a high concentration of CsA (10 µg/ml, $p < 0.05$, **Figure 3H**).

Proliferation and Differentiation of the Treg Subpopulation Is Highly Perturbed by EC Preexposure to Immunosuppressors or to IVIg

We next determined the effect of MPA, CsA, or IVIg on the Treg population. This population was defined as $CD4^+CD45RA^{neg}FoxP3^{bright}$ (Gating strategy shown in Figure S5 in Supplementary Material), and are also $CD25^+$ and $CD127^{low}$. The proportion and the proliferation of Treg was strongly diminished by EC preincubation with either MPA or CsA (**Figures 4A–D**). The proportion of Treg was reduced to 65% of the proportion expanded by control EC ($p < 0.01$), and the percentage of proliferating Treg was reduced to 11.4% ($p < 0.05$) in the presence of CsA-treated EC. These decreases were selective for Treg as the Th17 subpopulation was unaltered by EC preincubation with CsA. Proliferation of existing Treg has been identified as a mechanism of Treg amplification; the decrease in Treg induced by MPA and CsA may therefore be due to reduced proliferation (**Figures 4B,D**).

These results demonstrate that EC exposure to immunosuppressors skews their orientation of the alloimmune response. CsA biased toward a proinflammatory profile by lowering Treg and modestly increasing Th1 (**Figure 3E**). Exposure of EC to MPA also biased toward a proinflammatory profile by reducing Treg despite an unchanged Th17 response.

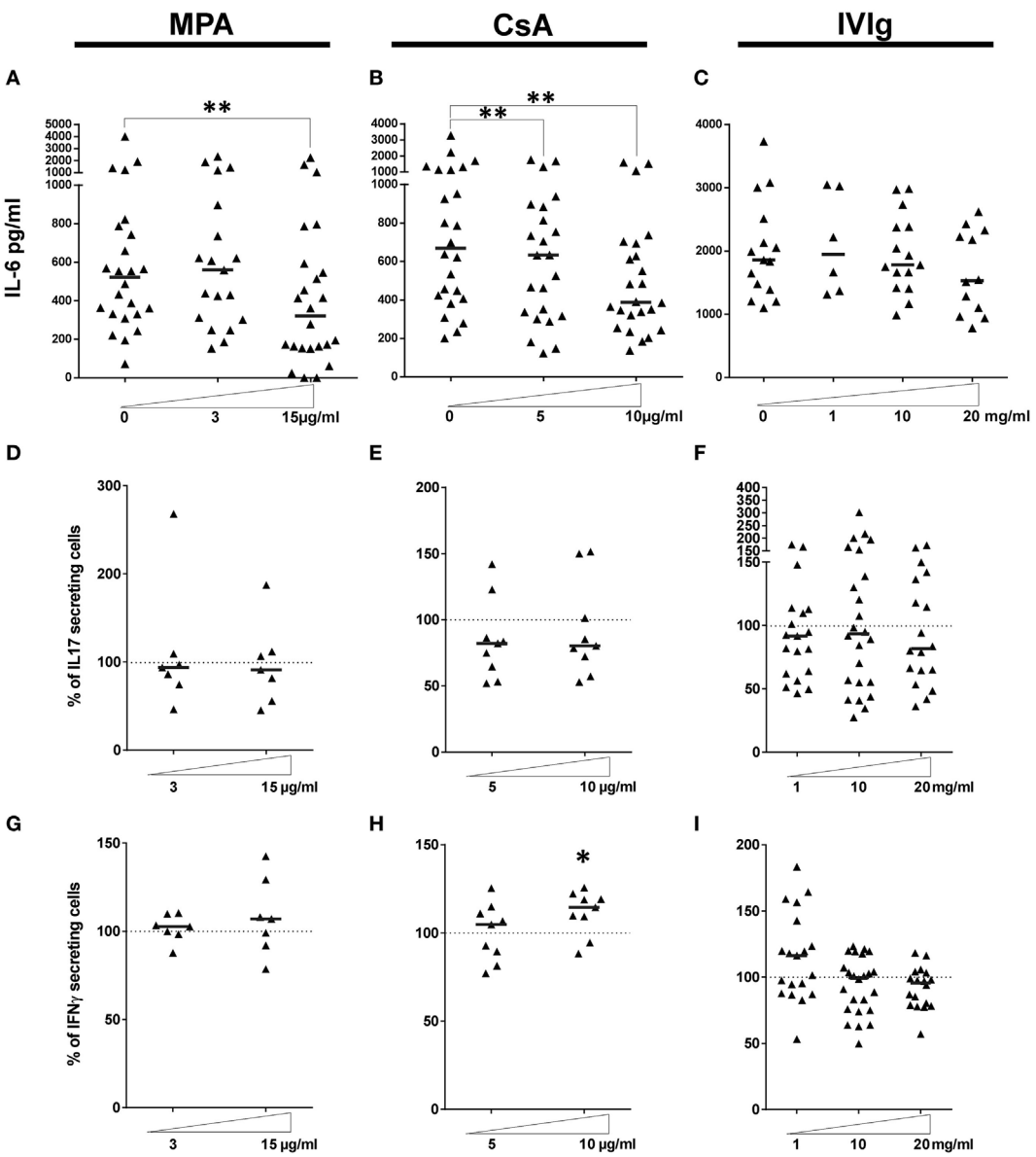

FIGURE 3 | Effect of endothelial cell (EC) incubation with mycophenolic acid (MPA), cyclosporine A (CsA), and intravenous immunoglobulin (IVIg) on the proinflammatory activity of ECs. IL-6 secretion was quantified in the supernatants of cocultures where ECs was treated with MPA [**(A)**, $n > 19$ donors], CsA [**(B)**, $n = 24$ donors] or IVIg [**(C)**, $n > 6$ donors]. IL-6 production by ECs incubated with vehicle alone is represented as 0. **(D–F)** show the expansion of the Th17 subset after a 7 day coculture of PBMC with EC pretreated with interferon γ (IFN-γ) and the indicated dose of MPA [**(D)**, $n = 7$ donors], CsA [**(E)**, $n = 9$ donors], or IVIg [**(F)**, $n > 18$ donors]. Expansion of the Th1 subset under the same conditions is shown in [**(G)**, $n > 7$ donors], [**(H)**, $n = 9$ donors], and [**(I)**, $n > 18$ donors]. Results are expressed as the relative percentage of the control values (ECs treated with vehicle alone, represented by dotted lines). Thick, horizontal lines represent median values in all the cocultures (*$p < 0.05$, **$p < 0.01$: two-tailed Wilcoxon paired test).

Endothelial cell expression of HLA-DR was necessary and sufficient for $CD4^+$-T cell proliferation and differentiation (35) and IVIg was the only immunomodulator tested that increased HLA-DR expression (respective median values of 155.1 and 113.9% with 1 and 10 mg/ml IVIg, **Figure 1F**). **Figure 4E** reveals increased Treg differentiation after preincubation of EC with IVIg. In addition, the proliferation of Treg cells was increased to a median value of 130.4% ($p = 0.005$) by IVIg-treated ECs (**Figure 4F**).

These data are consistent with findings from other investigators of increased Tregs after IVIg treatment in murine models of allergic airways disease or EAE (22, 26) and in the context of human autoimmune pathologies. However, in these studies, differentiation of tolerogenic DC after treatment with IVIg was identified as the mechanism of IVIg mediated Treg expansion. In the current model, only the EC were stimulated with IVIg (22) prior to coculture with PBMC from allogeneic donors.

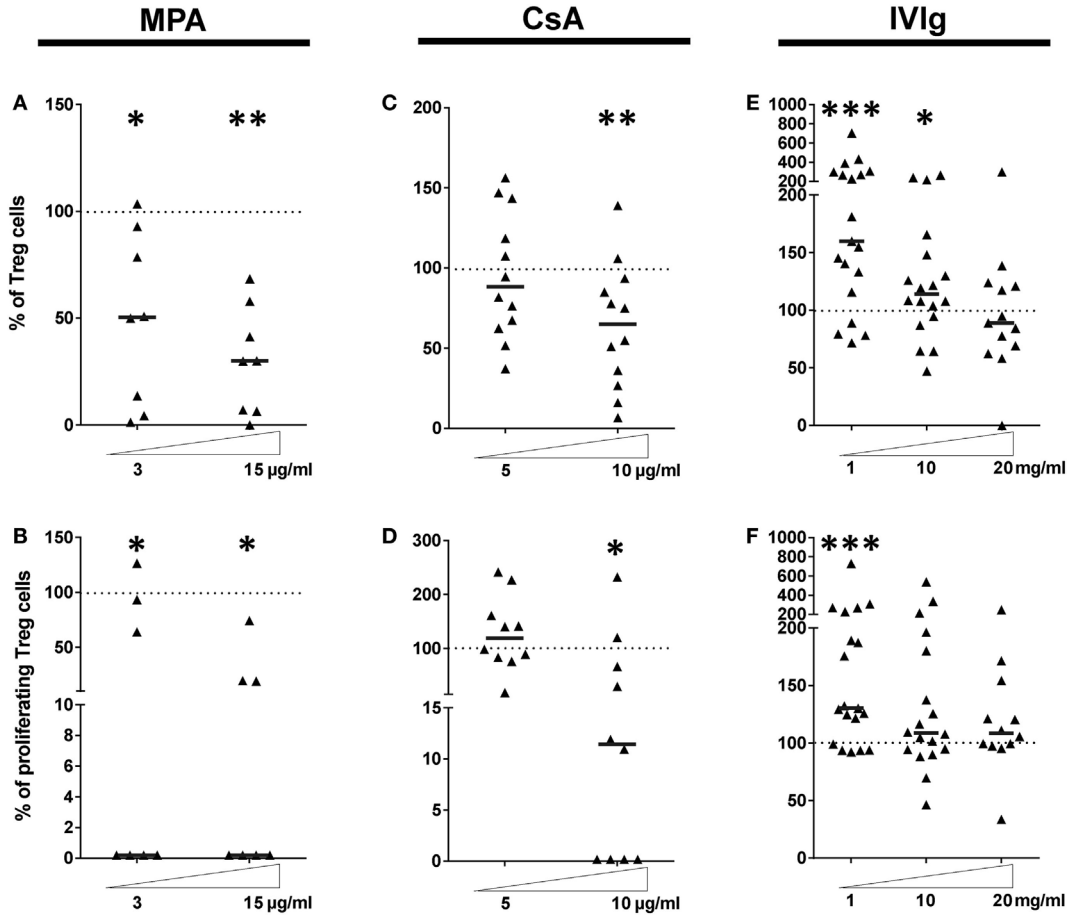

FIGURE 4 | Intravenous immunoglobulin (IVIg) pretreatment of endothelial cells (ECs) induces selective amplification of regulatory T cells in contrast with the commonly used immunosupressors mycophenolic acid (MPA) and cyclosporine A (CsA). **(A,C,E)** show the proportion of Treg expanded in cocultures of PBMC with EC pretreated with interferon γ (IFN-γ) and the indicated concentrations of MPA [**(A)**, $n = 8$ donors], CsA [**(B)**, $n = 12$ donors], or IVIg [**(C)**, $n > 13$ donors]. [**(D)**, $n = 7$ donors], [**(E)**, $n = 10$ donors], and [**(F)**, $n > 12$ donors] show the proliferation of Treg after MPA, CsA, or IVIg treatment of ECs, respectively. Results are expressed as the relative percentage of the control values (ECs treated with vehicle alone and represented by dotted lines). Thick, horizontal lines show the median values in each data set (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$: two-tailed Wilcoxon paired test).

Exposure of PBMC to Immunosuppressors or to IVIg Does Not Prevent Altered Generation of CD4$^+$-T Cell Subsets by EC Which Were Pretreated with MPA, CsA, or IVIg in Comparison with Non-Treated EC

In vivo therapeutic use of immunomodulators would target both EC and PBMC. It was therefore important to examine whether expansion of CD4$^+$-T cell populations, modified by pretreatment of ECs, were maintained after exposure of PBMC to either immunosuppressors or IVIg. The data in **Figure 5** show that the effects of MPA, on EC-mediated expansion of Tmem, and Treg were conserved after exposure of PBMC to the different treatments. Pretreatment of EC and PBMC with MPA resulted in less Tmem and Treg proliferation than when only PBMC were pretreated with MPA (**Figures 5C–F**). The lower level of Treg proliferation was particularly marked (**Figure 5F**). In contrast, neither proliferation nor amplification of Th17 or Th1 was modified by MPA (**Figures 5A,B**). Additionally, although neither the proportion nor the proliferation of Tmem was altered by PBMC and EC exposure to CsA (**Figures 5C,D**), reduced Treg as well as reduced Treg proliferation, in comparison with PBMC alone, was observed (**Figures 5E,F**). Moreover, the proportion of Th17 cells was enhanced under these conditions (**Figure 5A**).

Regarding EC pretreatment with IVIg, Tmem, Th17, nor Th1 (**Figures 5A–D**) were significantly altered by preexposure of EC and PBMC to IVIg whereas the proportion of Treg was increased compared with Treg from pretreated PBMC alone (**Figure 5E**). Enhancement of Treg proliferation and differentiation by a direct action of IVIg on EC is therefore sustained when PBMC have also been exposed to IVIg prior to coculture with EC.

The overall equilibrium between the major CD4$^+$-T cell subsets expanded by EC in this model was then determined. **Figure 6** reveals the change in the ratio of Treg:Th17 relative to the initial ratio of Treg:Th17 amplified in the presence of non-treated EC.

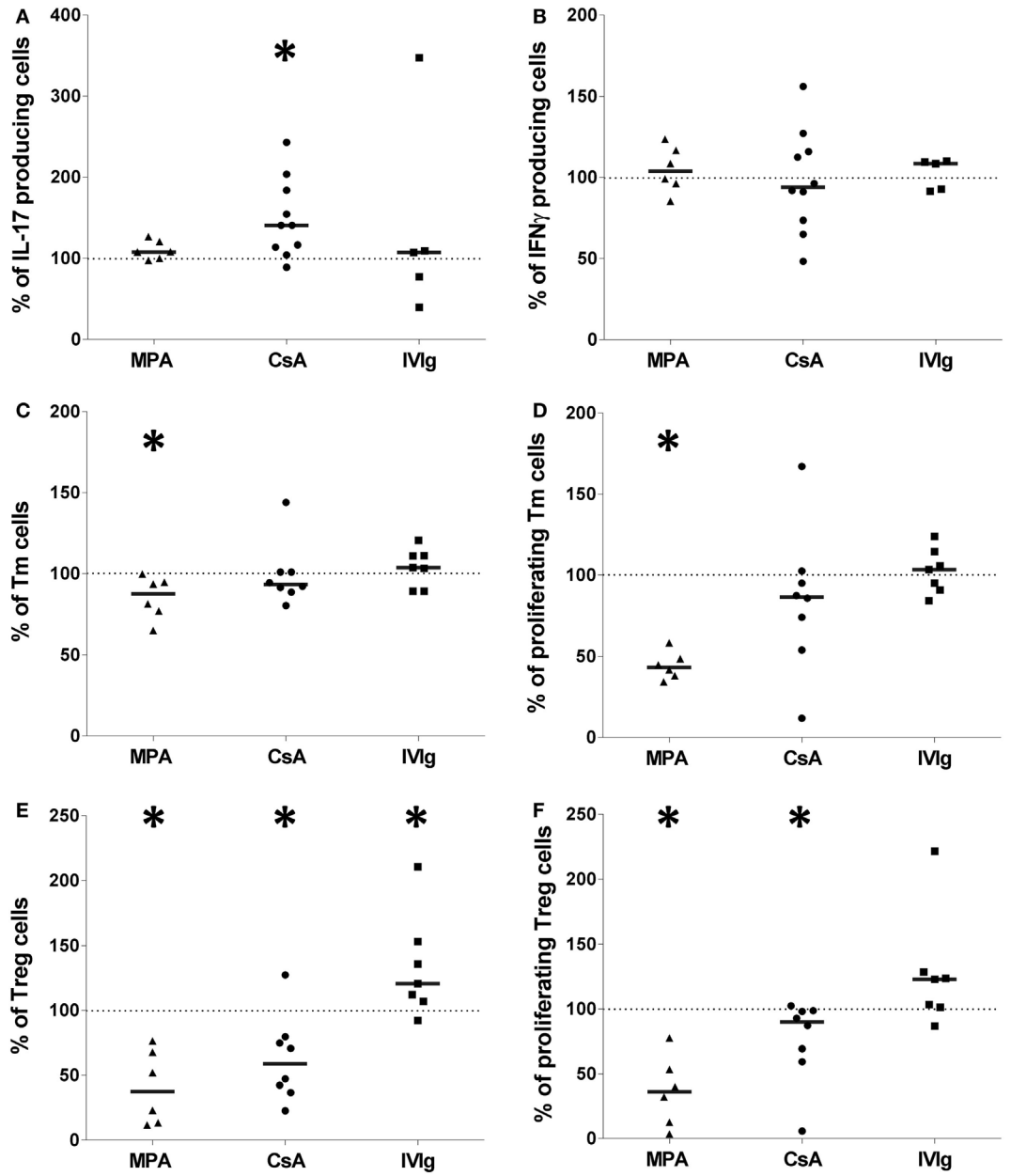

FIGURE 5 | The effect of mycophenolic acid (MPA), cyclosporine A (CsA), or intravenous immunoglobulin (IVIg) on endothelial cell (EC)-mediated allogeneic $CD4^+$-T cell polarization is maintained when PBMC have been exposed to immunosuppressors or IVIg. Figure shows the proportion of interleukin-17 producing cells [**(A)**, $n > 5$ donors], interferon γ (IFN-γ) producing cells [**(B)**, $n > 5$ donors], T memory cells [**(C)**, $n > 6$ donors], and their proliferation [**(D)**, $n > 6$ donors], Treg [**(E)**, $n > 6$ donors], and Treg proliferation [**(F)**, $n > 6$ donors]. Like ECs, PBMC were pretreated with MPA, CSA, or IVIg prior to coculture. In all conditions, PBMC and ECs were treated with the same immunomodulators. The concentrations used for stimulation of ECs and PBMC were 3 µg/ml for MPA, 5 µg/ml for CsA, and 1 mg/ml for IVIg. Results are expressed as the relative percentage of the control values corresponding to ECs treated with vehicle and PBMC treated by immunomodulators (represented by dotted lines). In all figures, horizontal lines show median values (*$p < 0.05$: two-tailed Wilcoxon paired test).

While MPA decreased the proportion of Treg:Th17, CsA did not significantly change the ratio at either concentration tested. In contrast the IVIg treatment of EC led to a two- to threefold increase in the ratio of Treg:Th17. Together these results indicate that EC are a target for either immunosuppressors or for IVIg and that the effect on ECs is maintained when PBMC have been exposed to MPA, CsA, or IVIg. These data lead to the suggestion that EC may contribute to the overall immunosuppression observed *in vivo* and as well as to the protolerance effect observed after treatment of allosensitized patients to IVIg.

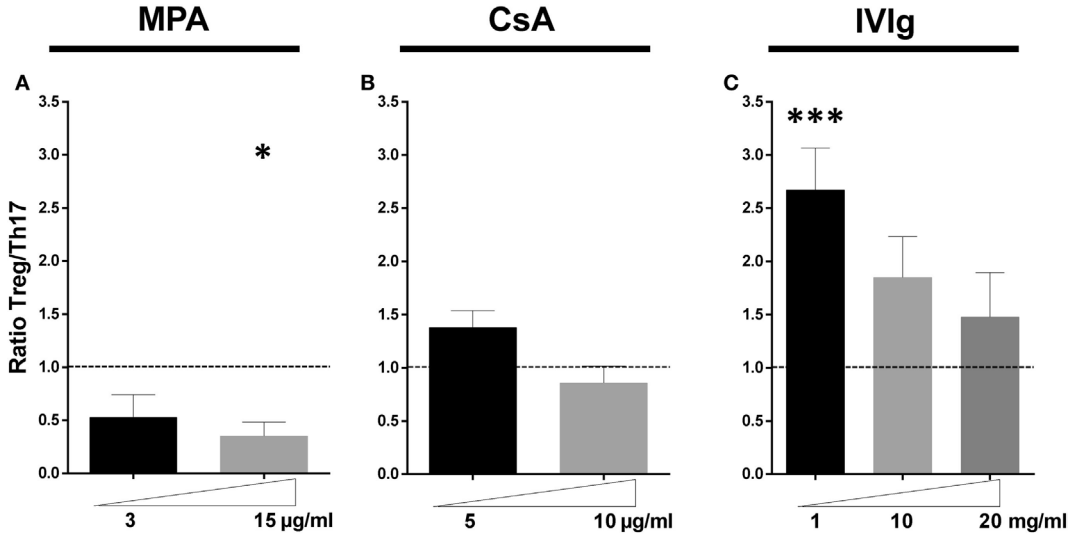

FIGURE 6 | Intravenous immunoglobulin promotes a proregulatory environment in contrast to mycophenolic acid (MPA) and cyclosporine A (CsA). The ratio of the initial proportion of Treg:Th17 in the absence of MPA, CsA, or intravenous immunoglobulin (IVIg) was normalized to 1 (represented by dotted lines) for each donor and the change in this ratio after pretreatment of endothelial cell (EC) with the forementioned immunomodulators is shown. Ratio of Treg: IL-17 producing cells in cocultures of PBMC and ECs pretreated as indicated with MPA [**(A)**, $n = 7$ donors], CsA [**(B)**, $n = 8$ donors], or IVIg [**(C)**, $n > 12$ donors] is shown. For all graphs, the mean ± SEM are indicated (*$p < 0.05$, ***$p < 0.001$: two-tailed Wilcoxon paired test).

Effects of Combining CsA or MPA with IVIg

Mycophenolic acid and CsA belong to distinct classes of immunosuppressors, MPA targets nucleotide synthesis while CsA targets calcineurin. IVIg is distinct from both but is often used in combination with one or other. Both MPA and CsA had similar effects on EC phenotype and induced a reduction of $CD4^+CD45RA^{neg}FoxP3^{bright}$ Treg cells. This was in marked contrast to the results of IVIg treatment. We therefore tested the outcome of EC pretreatment with immunosuppressors of different classes and IVIg: MPA and IVIg or CsA and IVIg. When ECs were pretreated with either MPA and IVIg or CsA and IVIg, the increased expression of CD54 observed with either immunosuppressor alone (confirming data in **Figure 1**; Figure S1 in Supplementary Material) was further enhanced by combining them with IVIg (**Figures 7A,B**).

Concerning HLA-DR expression, MPA alone decreased expression (confirming the data in **Figure 1**; Figure S1 in Supplementary Material). We also observed a higher level of HLA-DR expression in cells which had been treated with either concentration of IVIg and MPA in comparison with cells treated with MPA alone (**Figure 7C**). With the combination of IVIg and CsA, compensation of the loss of HLA-DR expression was not observed (**Figure 7D**) (unlike the combination of IVIg and MPA).

As detected for CD54 expression, associated immunomodulators had a cumulative effect on the reduction of IL-6 secretion by ECs (**Figures 7E,F**). When the IL-6 production was determined in cocultures of PBMC with ECs, which had been pretreated with combinations of immunosuppressors, no significant difference in the IL-6 production was observed in comparison with MPA or CsA pretreatment alone (**Figures 7G,H**).

DISCUSSION

Although endothelial lesions within the allograft have been long recognized and indeed used as a marker of allograft damage, the role of the endothelium as a mediator of the alloimmune response has recently become the object of intense study (42, 47, 48). The current study addressed whether the commonly used immunosuppressors, MPA and CsA or a regularly used treatment for alloimmunized patients, IVIg, modified endothelial allogenicity.

The results demonstrate that these immunomodulators, used to promote allograft tolerance, act directly upon the phenotype, cytokine secretion and allogeneic function of human ECs in an inflammatory environment. These data are summarized in **Table 1**. The immunosuppressors, CsA and MPA, promoted EC allogenicity toward a proinflammatory $CD4^+$-T cell response by decreasing the amplification of Treg by ECs. In contrast, IVIg, used in the desensitization of alloimmunized transplant patients, increased the generation of a regulatory response. Modified expression of CD54 and HLA-DR, both at the mRNA and at the protein level, and altered IL-6 secretion, were observed following exposure of EC to the above immunomodulators. When MPA or CsA were combined with IVIg in order to better imitate the conditions of therapeutic use, IVIg could act to partially compensate the HLA-DR decrease mediated by MPA, while the increase in CD54 expression was further increased when IVIg was present.

Generation of a pro- or anti-inflammatory response was determined in a model of the interaction between microvascular EC and PBMC from non-related donors. Although the experimental set-up is an imperfect model, it recapitulates key aspects of *in vivo* interactions in the allograft microvasculature firstly because human EC activate allogeneic $CD4^+$-T effector memory

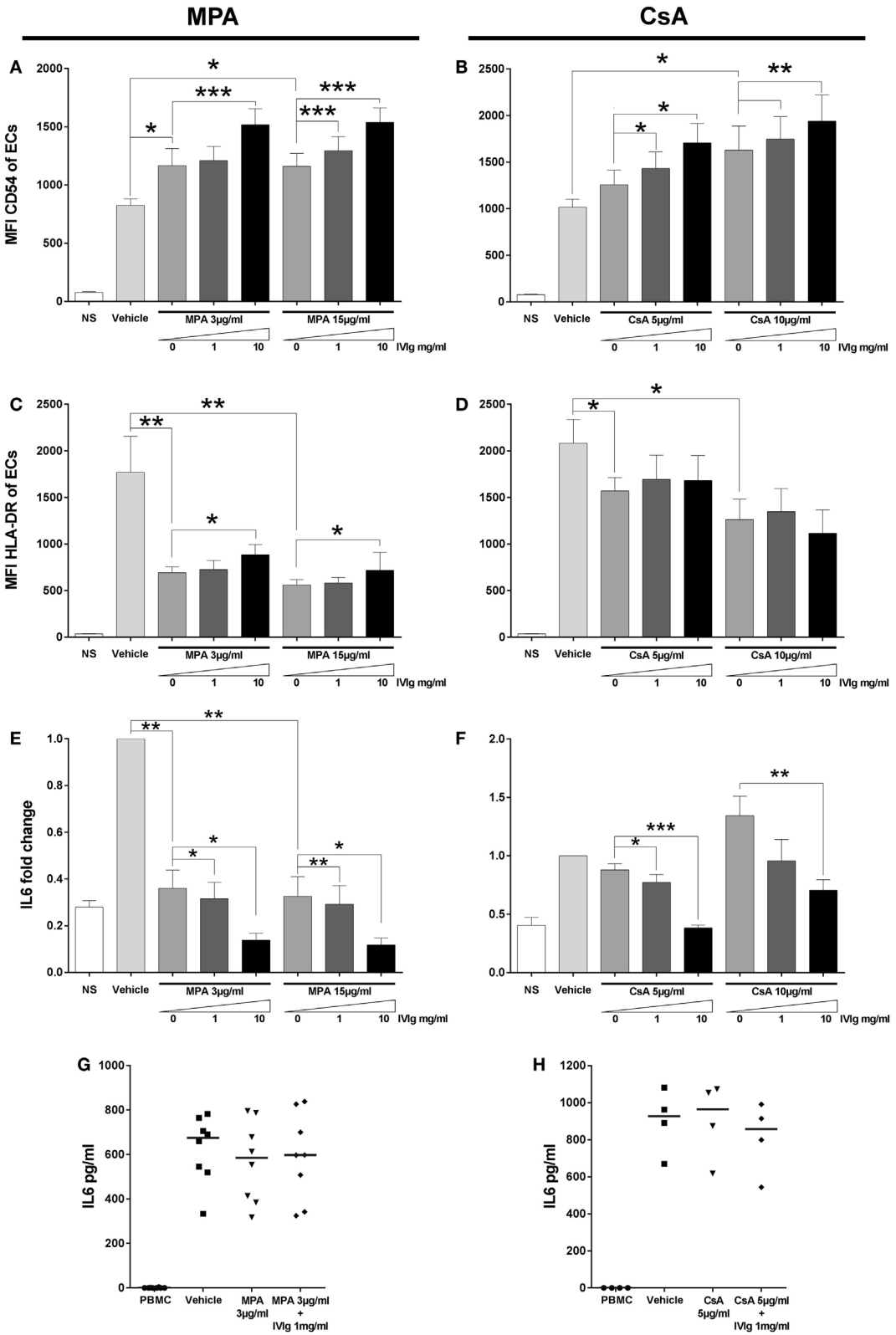

FIGURE 7 | Continued

FIGURE 7 | Continued
Addition of Intravenous Immunoglobulin to immunosupressors modifies their impact on endothelial cell (EC) phenotypes and interleukin (IL)-6 secretion by EC alone or in coculture. The phenotype of ECs was studied by flow cytometry after 3 days of treatment with interferon γ and the indicated combinations of immunomodulators. NS represents the "non-stimulated" condition. The control vehicle is methanol or ethanol for combinations of mycophenolic acid (MPA) with intravenous immunoglobulin (IVIg) or for combinations of cyclosporine A (CsA) with IVIg, respectively. The mean CD54 fluorescence intensity (MFI) is shown after treatment of cells with MPA and IVIg [**(A)**, $n = 4$] or with CsA and IVIg [**(B)**, $n = 4$]. [**(C)**, $n = 5$] and [**(D)**, $n = 5$] show the MFI of HLA-DR after treatment of ECs, respectively, with MPA and IVIg or CsA and IVIg. IL-6 secretion by ECs was quantified and represented as fold change of the level produced by cells incubated with vehicle alone in [**(E)**, $n = 4$] and [**(F)**, $n = 4$] where ECs was treated with MPA and IVIg or with CsA and IVIg, respectively. Finally, [**(G)**, $n = 8$] and [**(H)**, $n = 4$] show the effect on IL-6 secretion after treatment of ECs with MPA and IVIg or with CsA and IVIg, respectively, in coculture with PBMC. Horizontal columns show mean values ± SEM (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, paired *t*-test).

TABLE 1 | Summary of immunosuppressor-mediated changes in endothelial cell (EC) phenotype and in the downstream changes in allogeneic T lymphocyte polarization.

		Mycophenolic acid	Cyclosporine A	Intravenous Immunoglobulin (IVIg)
Direct changes to endothelial phenotype	Surface CD54 expression	↑	↑	↑
	Surface HLA-DR expression	↓	↓	↑
	IL-6 secretion	↓	↓↑	↓
Indirect changes to T lymphocyte polarization	% of $CD4^+$ T memory cells	=	=	=
	Alloproliferation of memory T cells	↓	=	=
	IL-6 secretion	↓	↓	=
	% of Th1	=	↑	=
	% of Th17	=	=	=
	% of Treg	↓	↓	↑[a]
	% of Treg proliferation	↓	↓	↑[a]

Changes shown are relative to non-treated controls.
[a]*Highest increases were observed at the concentration of 1 mg/ml IVIg.*

cells while murine ECs only activate a regulatory response and secondly because it allows monitoring of $CD4^+$-T lymphocyte subpopulations relevant to the *in vivo* situation in transplant patients (32, 49).

HLA class II antigens are expressed in the steady state by human ECs and the level is strongly increased under inflammatory conditions (30, 31). Such expression can lead to activation and proliferation of allogeneic T effector memory cells (32) and to selective amplification of Th17 and Treg populations (35). Activation of proinflammatory $CD4^+$-T by HLA-DR expressing ECs is further increased by EC activation with alloantibodies (42). The current study identifies new and clinically relevant regulation of EC induced pro- or anti-inflammatory responses by the immunosuppressors CsA and MPA or the immunomodulator IVIg. Moreover, the mechanism relied upon the direct activity of CsA, MPA, or IVIg on the EC.

Non-identical changes in EC phenotype were observed in the presence of CsA and MPA although both oriented the $CD4^+$-T cell response toward a proinflammatory profile. For example, although HLA-DR expression was decreased by both CsA and MPA, the reduction was more drastic in the presence of MPA. This is probably due to their different targets, CsA is a prototypical calcineurin inhibitor, whereas MPA acts directly upon nucleotide synthesis. CsA has multiple effects and in an allogenic tracheal transplantation model in mice, CsA prevented $CD8^+$-T cells infiltration into the graft and limited the Th1 response (50). In the current model, the effect of MPA on the cell surface expression of CD54 and HLA-DR was concordant with its effect on mRNA levels indicating that the regulation may proceed by a transcriptional mechanism. This was in contrast with CsA that did not alter mRNA levels of HLA-DR or CD54 in the same way as observed at the cell surface.

While CsA and MPA have different targets, and probably different mechanisms of altering the EC phenotype and function (suggested by the differential regulation of HLA-DR and IL-6), both enhanced the proinflammatory response, this increase may be implicated in long-term effects of CsA and MPA. IVIg differed from both CsA and MPA by increasing HLA-DR expression and by decreasing IL-6 secretion in a dose-dependent manner both at the level of the protein and of mRNA.

Although MPA and IVIg acted individually to reduce IL-6 secretion, only MPA and CsA decreased IL-6 in cocultures of EC with PBMC. The molecular interactions leading to higher IL-6 secretion by EC cocultured with allogeneic PBMC have not been identified but they may be targets of either MPA or CsA in the EC.

Intravenous immunoglobulin contrasted with both CsA and MPA by increasing the differentiation of Treg and particularly at the lowest concentration of IVIg tested. It is interesting to note that increased differentiation of Treg in response to human EC has previously been reported in the presence of Rapamycin. The mechanism was dependant on expression of the costimulatory molecule PDL1 (51). Although PDL1 is also expressed in our EC model, it was unaltered by exposure to CsA, MPA, or IVIg (data not shown). Moreover, in a previous study, we have not observed a role for PDL1 in Treg expansion (35).

The loss of the capacity for Treg differentiation by ECs exposed to CsA or MPA is striking. This may result from the decreased

expression of HLA-DR, we have previously reported that HLA-DR expression is necessary and sufficient for T cell proliferation in this model and that Treg expansion relied upon proliferation (35). The importance of Treg in the transplant setting is well documented. In rodent models, Tregs significantly prolong the survival of skin (52, 53) and heart (54, 55) allografts. Correlations between the proportion of Tregs within allografts and graft survival have been observed in humans (38, 40).

The current study employed immunosuppressors and IVIg at concentrations that are close to the estimated circulating levels in transplanted patients. However, this is an approximation and even in the *in vivo* setting, exact concentrations within the graft microvasculature may not reflect those in the circulation. This may be a reason for the preferential amplification of Treg at the lower concentrations of IVIg tested. This study primarily addressed the role of the individual effects of MPA, CsA, and IVIg although these are most often administered in combinations or with corticosteroids. It will now be necessary to fully identify the pathways activated by IVIg, CsA, or MPA in ECs individually and in association.

Results of the experiments with combinations of either MPA and IVIg or CsA and IVIg underline the potential differences in their mechanisms. IVIg associated with MPA somewhat compensated the effect of MPA on HLA-DR expression, although the effect of MPA was dominant. When CsA was tested in combination with IVIg, the reduction of HLA-DR expression with CsA alone was not compensated. However, this reduction was less than that obtained with MPA alone and this could explain the lack of visible effect of IVIg.

Regarding IL-6 production, the interaction between EC and PBMC increases IL-6 production by, as yet unidentified interactions (42). The effect of either MPA or CsA on IL-6 production by ECs alone was amplified by the addition of IVIg although this effect was not visible following EC interactions with PBMC. This may be due to the low concentrations of MPA and CsA tested in combination with IVIg. Together, these results also suggest that the influence of IVIg on CsA treatment has different consequences than observed with MPA and highlight the different mechanisms of regulation of HLA-DR expression induced by CsA or MPA treatment of ECs.

Finally, these data underline the possible long-term effects of CsA and MPA in promoting EC induced proinflammatory responses. This is in clear contrast to the potential role of IVIg in boosting the EC induced regulatory response.

AUTHOR CONTRIBUTIONS

JL, DG, and NM designed the study and analyzed and interpreted data. JL, MB, AC, and KP acquired, analyzed, and interpreted data. AH, CT, and SK contributed to the study design. JL and NM wrote the manuscript. All authors approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01761/full#supplementary-material.

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Transplantation Tolerance through Hematopoietic Chimerism: Progress and Challenges for Clinical Translation

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The perception that transplantation of hematopoietic stem cells can confer tolerance to any tissue or organ from the same donor is widely accepted but it has not yet become a treatment option in clinical routine. The reasons for this are multifaceted but can generally be classified into safety and efficacy concerns that also became evident from the results of the first clinical pilot trials. In comparison to standard immunosuppressive therapies, the infection risk associated with the cytotoxic pre-conditioning necessary to allow allogeneic bone marrow engraftment and the risk of developing graft-vs.-host disease (GVHD) constitute the most prohibitive hurdles. However, several approaches have recently been developed at the experimental level to reduce or even overcome the necessity for cytoreductive conditioning, such as costimulation blockade, pro-apoptotic drugs, or Treg therapy. But even in the absence of any hazardous pretreatment, the recipients are exposed to the risk of developing GVHD as long as non-tolerant donor T cells are present. Total lymphoid irradiation and enriching the stem cell graft with facilitating cells emerged as potential strategies to reduce this peril. On the other hand, the long-lasting survival of kidney allografts, seen with transient chimerism in some clinical series, questions the need for durable chimerism for robust tolerance. From a safety point of view, loss of chimerism would indeed be favorable as it eliminates the risk of GVHD, but also complicates the assessment of tolerance. Therefore, other biomarkers are warranted to monitor tolerance and to identify those patients who can safely be weaned off immunosuppression. In addition to these safety concerns, the limited efficacy of the current pilot trials with approximately 40–60% patients becoming tolerant remains an important issue that needs to be resolved. Overall, the road ahead to clinical routine may still be rocky but the first successful long-term patients and progress in pre-clinical research provide encouraging evidence that deliberately inducing tolerance through hematopoietic chimerism might eventually make it from dream to reality.

Keywords: tolerance, chimerism, allograft rejection, transplantation immunology, immunosuppression

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Abbreviations: ATG, α-thymocyte globulin; HLA, human leukocyte antigen; MHC, major histocompatibility complex; NK, natural killer; NKT, natural killer T cells; TLI, total lymphoid irradiaton; TBI, total body irradiation; Treg, regulatory T cells.

THE LONG-LASTING JOURNEY OF TOLERANCE

Transplantation is the treatment of choice for patients with end-stage organ failure (1, 2) as it improves their survival and quality of life (3). Nevertheless, long waiting lists, side effects of immunosuppressive medication, and limited graft survival still perpetuate the dream of tolerance. Initial enthusiasm was fueled in the 1950s by the observation of Medawar and Billingham that dizygotic twins of free martin cattle readily accept skin grafts from each other (4). They attributed this phenomenon to the coexistence of red blood cells from their siblings (mixed chimerism) as a result of a common placental circuit (5). Since fraternal erythrocytes also persisted in adult animals, they concluded that stem cells had to be exchanged which constantly give rise to short-lived red blood cells. Based on this observation, they demonstrated for the first time that tolerance to alloantigens can actively be acquired in neonatal mice by the intrauterine injection of allogeneic cells (6). Since then, a myriad of mouse studies established bone marrow transplantation as promising approach to achieve donor-specific transplantation tolerance (7). In the clinical setting, the usability of this approach was corroborated by anecdotal cases in which patients developed immunological tolerance to a renal allograft after having previously received a hematopoietic stem cell transplant from the same donor for a hematological disorder (8). Despite this knowledge, the establishment of tolerance inducing protocols as a clinical routine is still long in coming.

Three groups in the US from Harvard University, Stanford University and the association between the Universities of Louisville and Northwestern have pioneered the first steps toward clinical implementation. All three groups have elaborated distinct approaches with individual assets and drawbacks and so far, nearly 70 patients have been enrolled (9–11). Here, we discuss the current state of affairs of chimerism-based tolerance, what we have learned so far and which new challenges we have to face.

TOLERANCE—IS IT WORTH IT?

Continuous suppression of the immune system puts transplant recipients at risk of increased morbity and mortality through cardiovascular disease, *de novo* diabetes, dyslipidemia, and malignancies. By now, death with a functioning graft has become a leading cause of graft loss (2). Some of these side effects result from impaired immune surveillance while others constitute drug-specific toxicities of the immunosuppressive medication. Cyclosporine was initially celebrated as "wonder drug," until it was realized that it is fairly toxic at higher doses (12). In the light of this, a substantial number of non-renal organ transplant recipients develop renal failure due to calcineurin inhibitor-toxicity (13). Apart from that, immunosuppressive drugs are ineffective in preventing late graft loss from chronic rejection (14) which is why long-term graft survival has improved only marginally over the last decades (2).

Since tolerance is expected to provide remedy, the search for the "Holy Grail" of transplantation has never ceased. To assess whether tolerance actually meets these high expectations, tolerant kidney transplant recipients have recently been compared to a matched cohort receiving conventional immunosuppression. The tolerant group experienced significantly longer initial hospital stays and more frequent readmissions leading to three times higher costs during the first year in comparison to conventional transplant recipients. In turn, tolerant patients required significantly less treatment for hypertension and none of them developed new-onset diabetes, dyslipidemia, or malignancy. In this survey, the continuous costs for medications of conventional patients exceeded those of tolerant patients after 10 years (15, 16). The sample size was small but this preliminary study emphasized the potential benefit of tolerance inducing protocols. Another group estimated the expected lifetime savings through tolerance induction for a 40-year old patient receiving a kidney from a human leukocyte antigen (HLA)-matched living donor to approximately 92.000$ (17). Besides, it should be taken into account that innovative treatment options become less expensive if employed as clinical routine, especially if they are also applicable to other medical fields. In this regard, mixed chimerism becomes increasingly attractive as treatment option for autoimmune disorders (18, 19).

In addition, tolerant patients evidently enjoy a higher quality of life (16), which is not only a matter of convenience but also correlates with reduced morbidity and mortality (20). The discomfort resulting from the immunosuppressive therapy increases the probability of non-adherence which in further consequence leads to decreased donor organ survival. Considering that kidney transplant recipients take a median of 15 capsules a day (21), it appears not surprising that non-adherence is estimated to occur roughly in a third of all transplant recipients (2).

Tolerance inducing protocols are currently measured against kidney transplant recipients receiving conventional immunosuppressive therapy. One-year graft survival rates of over 90% and half-lives of 16 years set the bar fairly high (22, 23). Innovative strategies aiming to improve patient and organ survival will further increase the high demands for tolerance inducing strategies. New algorithms have been developed to allocate best-quality organs to those recipients expected with the highest survival (24) and paired living kidney donation programs optimize allograft survival (25). Furthermore, the long-lasting supremacy of calcineurin inhibitors has recently been challenged by the advent of costimulation blockers in the clinical setting. Belatacept was associated with enhanced overall patient and graft survival in subsets of patients, improved kidney allograft function and avoided major side effects of calcineurin inhibitors (26).

In summary, tolerance is still of great value, particularly in the longer run, but the question remains whether it can be achieved at an acceptable prize in terms of safety. Tolerance would certainly obviate the common risks of immunosuppressive medication but at the same time expose patients to other serious hazards.

GRAFT-VS.-HOST DISEASE (GvHD)—THE PROBLEM CHILD OF CHIMERISM

When it comes to clinical translation, patient safety takes the highest priority. Therefore, the conditioning necessary to

achieve allogeneic bone marrow engraftment is a matter of great concern. In contrast to conventional kidney allograft recipients, patients receiving hematopoietic stem cell transplantation for the purpose of tolerance induction are additionally exposed to a considerable risk of developing GvHD. Approximately 15% of patients undergoing hematopoietic stem cell transplantation for hematological diseases succumb to GvHD (27). The incidence and severity of acute GvHD can directly be correlated with the degree of HLA mismatch (28). Since tolerance is particularly desirable for HLA-mismatched recipients, the prevention and treatment of GvHD is a delicate issue. The occurrence of GvHD is highly dependent on the recipient conditioning and the composition of the allograft. Therefore, we will discuss which efforts the individual groups have made to minimize the risk of GvHD in the clinical pilot trials of chimerism-based tolerance.

Irradiation

Ionizing irradiation mostly affects mitotically active cells by causing breaks in DNA double strands. The cells of the hematopoietic system and the gastrointestinal tract exhibit a high degree of proliferation and are, thus, particularly sensitive to irradiation (29). Accordingly, high doses of total body irradiation (TBI) do not only obliterate the bone marrow compartment as required but also cause damage to the gastrointestinal tract. Bacterial molecules leaking from the injured gut elicit the release of inflammatory cytokines (e.g., TNF-α, IL-1, IL-6) through activation of innate immune receptors which promotes the induction of acute GvHD (30). In current clinical practice, the total dose is typically partitioned into lower doses to allow normal tissues to partially recover between the individual fractions (31). In addition, current effort is focused on the targeted neutralization of major inflammatory cytokines (32).

The clinical trials from the Stanford group are built upon a specific form of irradiation which was actually designed for the treatment of lymphomas (33). At this, irradiation is restricted to lymphatic tissues, including supradiaphragmatic lymph nodes, thymus, subdiaphragmatic lymph nodes, and spleen. Since a large part of the marrow volume is outside the radiation fields, recovery of blood elements occurs without severe neutropenia or thrombocytopenia (34). Total lymphoid irradiation (TLI) depletes the majority of lymphocytes within the targeted tissue end enriches the residual cells for $CD8^+$ dendritic cells and natural killer T cells (NKT) cells. $CD8^+$ dendritic cells prompt NKT cells to secrete IL-4 which prevents lethal GvHD through the expansion of donor Tregs (35, 36).

The group from Louisville adapted a conditioning regimen that was originally elaborated at the Johns Hopkins University for hematological disorders. This approach uses a single dose of 200 cGy TBI together with high-dose cyclophosphamide post-transplant to purge proliferating alloreactive T cells while sparing Tregs (37, 38). In this way, the increased proportion of donor Tregs prevents GvHD (39). Notably, the groups from Stanford and from Louisville both aim at increasing the number of donor Tregs to prevent GvHD. Donor Tregs have a vital role in reducing GvHD (40) and the infusion of donor Tregs has likewise been reported to prevent GvHD in the clinic (41).

The group from Boston attempted to reduce irradiation-related toxicities by specifically targeting those mechanisms resisting allogeneic bone marrow engraftment. In the murine setting, it was observed that depleting T cells with monoclonal antibodies allowed reducing myeloablative TBI. However, chimerism remained transient unless high doses of TBI were used (42), since these monoclonal antibodies efficiently depleted T cells in the periphery but did not reach T cells in the thymus. Hence, they combined T cell depleting antibodies with the targeted irradiation of the thymus to further decrease the required TBI to a non-myeloablative dose (43). Despite being successful in the murine setting, this regimen failed to induce stable mixed chimerism in non-human primates but instead led to transient chimerism. Nevertheless, the conditioning was sufficient to achieve tolerance to renal allografts across major histocompatibility complex (MHC) barriers as long as the kidney was transplanted before the loss of peripheral chimerism (44).

To exploit hematopoietic stem cell transplantation as clinical routine for tolerance induction it would be desirable, or indeed necessary, to avoid any form of irradiation. Several approaches have been elaborated in the murine setting to eliminate the cytotoxic preconditioning necessary to achieve allogeneic bone marrow engraftment. Costimulation blockade in the form of α-CD40L and CTLA4-Ig can only obviate the need for cytoreductive conditioning when clinically unrealistic marrow doses are administered (45, 46). The required bone marrow dose can be reduced through the addition of rapamycin or α-LFA-1 to the conditioning (47, 48). By contrast, conventional, clinically obtainable, doses of fully allogeneic bone marrow engraft in non-irradiated mice under costimulation blockade and rapamycin if *in vitro* activated Tregs from the recipient are administered at the time of donor bone marrow transplantation (49). An adjusted version of this approach has recently confirmed the feasibility of Treg therapy to enhance bone marrow engraftment in non-human primates (50). As clinical trials deploying Treg therapy without concomitant bone marrow transplantation are already underway, clinical translation of the combined cell therapy appears possible in the near future (51). Alternatively, the pro-apoptotic molecule ABT-737 synergized with α-CD40L and cyclosporine to induce chimerism and tolerance without the need for any cytoreductive conditioning (52). Both approaches, however, rely on CD40L blockade which is currently not available in the clinic due to unacceptable prothrombotic toxicities of conventional α-CD40L mAbs (53). In order to circumvent the unacceptable side effects of the original conventional α-CD40LmAbs, efforts were made to target its binding partner CD40 which is not expressed on thrombocytes. The humanized α-CD40 antibody ASK1240 has already been tested in a phase 2 trial of *de novo* kidney transplant recipients. The preliminary results, however, suggest a disappointing efficacy of CD40 blockade in a calcineurin inhibitor free regimen (54). Recently, next generation α-CD40L antibodies lacking thromboembolic side effects have shown promise in preclinical development and might become an option for use in tolerance protocols in the future (55).

Graft Composition

The identification of donor T cells as the driving force of GvHD conveyed the idea that transplanting purified stem cells could promote engraftment while avoiding GvHD. But it was soon realized that highly purified mouse stem cells would only engraft in MHC-matched but not -mismatched recipients (56). This failure of purified stem cells to engraft was traditionally ascribed to their rejection by host immune cells. However, it could also be envisioned that non-stem cell components contained within the donor bone marrow compartment are required to facilitate stem cell engraftment in allogeneic recipients. Therefore, the group from Louisville set themselves the task to prove this latter assumption and to identify such a cell population that facilitates bone marrow engraftment without causing GvHD. Adding selected donor cell populations to a mixture of T cell depleted syngeneic and allogenic bone marrow revealed a heterogeneous mixture of cells expressing $CD8^+$ albeit without a T cell receptor to promote stem cell engraftment (57). Further characterization of these murine "facilitating cells" unmasked plasmacytoid-precursor dendritic cells, B cells, granulocytes, as well as monocytes (58). Recipients of hematopoietic stem cells and "facilitating cells" displayed an increased RNA expression of GITR, CTLA4, and Foxp3 in the spleen 28 days post transplantation (59). In a subsequent study, the authors observed that $CD8^+$ plasmacytoid precursor DCs were primarily responsible for the induction of antigen-specific Foxp3 Tregs. These induced Tregs were able to enhance stem cell engraftment and to suppress alloreactive T cells *in vitro* (60).

Human "facilitating cells" are composed of two equally divided cell populations which can be differentiated on the basis of their CD56 expression. Most $CD56^{bright}$ cells are $CD11c^+$ $CD11b^+$ and exhibit a dendritic morphology. The majority of $CD56^{neg}$ cells expresses CD3ε and displays a lymphoid shape. Both cell populations express the chemokine receptor CXCR4 which promotes homing to the bone marrow compartment (61). Although it remains speculative, it is reasonable to assume that $CD8^+$ TCR^- $CD56^{bright}$ "facilitator cells" and $CD8^+$ DCs enriched through TLI share common features. The identification of this cell population constitutes the basic building block for the clinical trials of the Louisville/Northwestern group. A cell product containing hematopoietic stem cells and "facilitating cells" (also designated FCRx) is engineered through a proprietary, undisclosed procedure from G-CSF mobilized donor peripheral blood stem cells. The cell product is usually cryopreserved until it is infused 1 day after renal transplantation (62).

As already mentioned, the group from Stanford employed a regimen that was originally developed for patients with hematological malignancies. The key change for patients without malignancies was the alteration of graft composition in order to achieve mixed instead of full chimerism. While patients with malignancies received unmanipulated mobilized blood stem cells containing a high number of T cells ($2–3 \times 10^8$/kg), kidney recipients are transplanted with column enriched $CD34^+$ cells supplemented with low numbers of T cells (1×10^6/kg) (36). Under these circumstances kidney recipients have to be HLA matched in order to achieve stable mixed chimerism. Accordingly, this approach is only applicable to a restricted cohort of patients which enjoys anyhow best survival rates with current standard of care immunosuppressive treatment. However, the group from Stanford is currently conducting a clinical trial in the effort to determine the optimal graft composition for haploidentical donors (11).

The group from Boston transplants unseparated iliac crest marrow on the day of kidney transplantation, based on their experience from non-human primate studies (63). The acquisition of mobilized blood stem cells provides more comfort for the donors but mobilized blood stem cells have distinct biological characteristics that might affect their ability to induce tolerance. In a murine model, peripheral blood stem cells had a lower capacity to induce mixed chimerism and tolerance than conventional bone marrow due to the higher number of donor T cells (64), which can trigger rejection in an IL-6-dependent manner (65). From a clinical point of view, peripheral blood stem cell transplants are associated with a higher risk of acute and chronic GvHD but reduce the risk of graft failure owing to higher engraftment rates (66, 67). On the other hand, G-SCF mobilized stem cells upregulate CD47 to evade macrophage killing providing a possible explanation for their superior engraftment rates (68). Transplanting bone marrow and kidney at the same time requires the recipient conditioning to begin 6 days earlier which restricts this application to living donor transplant recipients. To extend their protocol to deceased donors the Boston group is currently endeavored to develop a "delayed tolerance" protocol. In this case, the recipients would first undergo kidney transplantation with conventional immunosuppression and subsequently receive cryopreserved bone marrow from the same donor. In non-human primates, kidney transplantation prior to bone marrow transplantation enhances the pool of alloreactive memory T cell responses, thus necessitating substantial CD8 T depletion to achieve mixed chimerism and tolerance (69, 70).

Chimerism Type

The type of chimerism (full vs. mixed, durable vs. transient) is a determining factor for the risk of GvHD. Murine studies indicated that there is a considerably greater chance of developing GvHD in stable full chimeras than in stable mixed chimeras (71). Apart from that, animal studies predict that stable mixed chimerism would also offer other advantages over full chimerism. The induction of mixed chimerism requires less toxic pre-conditioning and full chimeras display impaired immune responses resulting from the discrepancy between positive selection of T cells by host thymic epithelial cells and antigen presentation by peripheral donor antigen-presenting cells (72). However, the establishment of stable mixed chimerism in the clinic remains a formidable challenge. Therefore, it seems noteworthy to discuss the different forms of chimerism achieved by the individual groups.

The Northwestern group aims to achieve full chimerism which provides a stable state of tolerance although at the expense of a risk for GvHD. If stable chimerism is achieved, the patient is likely to maintain robust tolerance. On the downside, two cases of GvHD have been reported, one of which was fatal. Moreover, all patients had severe neutropenia (absolute neutrophil count <500 cells/mm^3) and 11 developed severe bacterial or fungal infections (10, 73).

The group from Stanford University aims to achieve stable mixed chimerism which is generally associated with a reduced risk of GvHD. However, thus far persistent mixed chimerism has solely been achieved in HLA-matched recipients who compromise only a minor patient cohort. Mixed chimerism is still difficult to achieve when HLA barriers are crossed. Anyhow, their conditioning showed a low incidence of adverse events as compared with other tolerance inducing strategies using cyclophosphamide and/or TBI (11).

The investigators from Boston consider GvHD as an inacceptable complication (44) for non-malignant patients and, therefore, intend to achieve tolerance through transient chimerism. Transient chimerism basically eliminates the risk of developing GvHD but exhibits a reduced stability of allograft tolerance. In non-human primate studies, 20–30% of the transiently chimeric recipients eventually developed antibody-mediated rejection during long-term follow-up (74). Similar complications occurred in the clinical trials wherefore the group had to adjust the protocol. Furthermore, all patients developed transient pancytopenia (75) and nine patients developed an engraftment syndrome with acute renal endothelial injury manifested by a creatinine rise during marrow recovery (76).

CURRENT STATE OF AFFAIRS

Boston Group

In their first trial, the group from Boston enrolled patients with end-stage renal disease resulting from multiple myeloma. These patients received a combined kidney and marrow transplantation from a HLA identical sibling donor. Ten recipients have been reported with follow-up times of up to 17 years. The conditioning consisted of cyclophosphamide (60 mg/kg; days −6 and −5), α-thymocyte globulin (ATG) (15–20 mg/kg; days −1, 1, 3, and 5) and thymic irradiation (700 cGy; day −1). Maintenance therapy in the form of cyclosporine was administered and discontinued as early as 73 days post-transplant in the absence of GvHD. Five patients have been completely off immunosuppression for 5–17 years providing the first proof of concept that tolerance in humans can deliberately be achieved through bone marrow transplantation. Recently, the inclusion criteria have been extended to other hematological disorders and cyclophosphamide has been replaced by 400 cGy TBI after severe cardiac toxicity occurred in one patient (77, 78).

The group from Boston modified the protocol in order to make it accessible also for patients with haploidentical donors. Cyclophosphamide was administered before transplantation at lower doses (14.5 mg/kg; days −6 and −5) and at higher doses (50 mg/kg; days 4 and 5) after transplantation. Thymic irradiation was substituted by 200 cGy TBI and cyclosporine by tacrolimus and mycophenolate mofetil. After the first patient experienced graft rejection, ATG was replaced by fludarabine (24 mg/m^2; days −6 to −2). The second patient tolerated the protocol fine, while the third patient deceased due to fludarabine-related neurotoxicity. Therefore, the dose of fludarabine was reduced from 5 to 3 days (24 mg/m^2/days −4 to −2) and the duration of individual dialysis sessions was extended. So far the transplant course of the forth patient was uncomplicated and he is off drugs 6 months post transplantation (77–79).

With the experience of the HLA-matched myeloma patients, the Boston group pursued their approach in patients without concomitant malignancies. The patients received a kidney and iliac crest marrow transplantation from haploidentical donors after thymic irradiation (700 cGy, day −1) and a short course of cyclophosphamide (60mg/kg/days −5 and −4). In contrast to myeloma patients, ATG was replaced by the monoclonal antibody Sipilizumab (α-CD2) to achieve more profound T cell depletion. The B cell depleting agent Rituximab (α-CD20) and prednisone were added after two patients developed donor-specific antibodies. Cyclosporine or Tacrolimus was slowly tapered over several months and completely discontinued at 8 months after confirming freedom from rejection by a 6-month protocol biopsy. All (10/10) patients developed transient chimerism and seven patients achieved tolerance from which four remain completely off immunosuppression (5–13 years) (9, 78, 80). To reduce the toxicity of the regimen and to ameliorate the engraftment syndrome cyclophosphamide has recently been substituted by TBI (2 × 150 cGy). So far two patients have been treated with this modified protocol, from which one remains off immunosuppression for more than 3 years. Future trials are expected to include the use of belatacept, based on murine (81, 82) and non-human primate studies (79, 80, 83).

Stanford Group

The approach from Stanford University is based on the observation that TLI was sufficient to prolong MHC-mismatched skin graft survival in mice and even to achieve long-term acceptance (>120 days) in combination with bone marrow cells (84). After successful establishment of this approach in larger animals (85, 86), the group went on to the clinical setting resulting in the first well-documented report of actively acquired immune tolerance in humans. Two recipients received 20 fractionated doses of TLI (100 cGy) and six doses of ATG (2 mg/kg; days 0, 2, 4, 6, 8, and 10), however, without hematopoietic stem cell transplantation (87).

Due to limited success rates and their experience from pre-clinical models, the group decided to add hematopoietic stem cell transplantation in 6 HLA-mismatched patients. After kidney transplantation (day 0), the patients received 10 doses of TLI (80–100 cGy; days 1–10) and 6 doses of ATG (1.5 mg/kg; days 0, 1, 3, 5, 9, and 14). CD34$^+$ stem cells (3.1–11.1 × 10^6) were column enriched from G-CSF mobilized blood stem cells, cryopreserved and administered 11 days after kidney transplantation. The cell product contained relatively low numbers of CD3$^+$ T cells (<0.1 × 10^6) to reduce the risk of GvHD. Maintenance therapy consisted of prednisone and cyclosporine. Two patients were weaned off immunosuppression after developing transient chimerism without signs of clinical rejection and exhibiting hyporesponsiveness to donor cells *in vitro*. Both patients developed rejection 3.5 and 5.5 months after withdrawal of immunosuppression. Increasing the dose of TLI did not improve chimerism (11, 88).

Thereupon the Stanford group focused on HLA-matched donor/recipient pairs deploying the same strategy with slight

modifications. Patients received 10 doses of 120 cGy TLI and 5 daily doses of ATG (1.5 mg/kg; days 0–4). All patients were infused with column enriched $CD34^+$ cells ($4.3–17.5 \times 10^6$/kg) supplemented with 1×10^6/kg $CD3^+$ T cells. Patients featuring stable chimerism for at least 6 months without signs of GVHD or clinical rejection were weaned off immunosuppression. 17 out of 22 patients were successfully tapered off immunosuppression. Seven patients exhibited stable mixed chimerism while the remaining 10 patients lost donor chimerism during or after withdrawal of cyclosporine. One patient with lupus had to return to maintenance immunosuppressive therapy after a systemic lupus flare.

Based on this success, the group recently reattempted their approach with haploidentical donor/recipient pairs. Currently, a dose escalation study is under way in order to determine the optimal dose of $CD34^+$ cells and $CD3^+$ T cells that would promote persistent mixed chimerism. Ten patients have been enrolled so far revealing that high levels of chimerism (at least 65% at 60 days) can be achieved with 50×10^6/kg $CD3^+$ cells and 10×10^6/kg $CD34^+$ cells. The ability of these patients to undergo successful drug withdrawal will be subject of a subsequent report with larger numbers of patients (11, 89).

Northwestern Group

The group from Northwestern University adopted a regimen for patients with non-malignant hematologic diseases and haploidentical donors (90). Kidney recipients received fludarabine (30 mg/m^2, days −4 to −2) together with dialysis, cyclophosphamide (50 mg/kg, days −3 and 3), and TBI (200 cGy, day −1). One day post kidney transplantation (day 0), a specially designed cell product (FCRx) engineered from G-CSF mobilized blood mononuclear cells is infused. Maintenance therapy in the form of Tacrolimus and mycophenolate mofetil is provided until a control biopsy is performed at 6 months. If the biopsy is clear, renal function is stable and more than 50% donor chimerism is present, immunosuppressive therapy is slowly tapered off. 30 of 31 patients exhibited donor chimerism at 1 month and 19 fulfilled the criteria for discontinuing maintenance therapy. The majority of the tolerant patients exhibited full chimerism (>98%) and three subjects mixed chimerism (10, 62, 73).

The investigators recently initiated a trial in which transplant recipients received multiple infusions of cryopreserved iliac crest and/or $CD34^+$ mobilized cells from HLA identical donors without myelosuppressive recipient conditioning, an approach reminiscent of previous bone marrow augmentation trials (91). Iliac crest bone marrow ($0.3–1.0 \times 10^6$) was infused 5 days post transplantation and GCSF-mobilized $CD34^+$ cells were infused 3, 6, and 9 months post transplantation. The recipients received two doses of Alemtuzumab (0.3 mg/kg; days 0 and 4) and maintenance therapy consisted of Tacrolimus and mycophenolate mofetil. No myelosuppressive conditioning was given and, consequently, no macrochimerism was induced, but rather only microchimerism was observed. After 3 months, Tacrolimus was replaced by Sirolimus (Rapamycin) and mycophenolate mofetil was discontinued between 12 and 18 months and Sirolimus after 24 months. Recipients were considered tolerant if they had normal biopsies and renal function after an additional 12 months without immunosuppression. Five of ten patients were successfully withdrawn from immunosuppression and showed normal protocol biopsies at 36 months. Tolerant patients exhibited transient chimerism for the first year and both tolerant and non-tolerant recipients exhibited increased proportions of $CD4^+CD25^{high}CD127^-FOXP3^+$ regulatory T cells and $CD19^+IgD/M^+CD27^-$ B cells (73, 92) (**Figures 1** and **2**; **Table 1**).

UNDERSTANDING THE MECHANISMS OF TOLERANCE

These first pilot trials provide the conceptual framework for the further development of chimerism-based approaches as clinically viable treatment option. In order to refine tolerance inducing strategies, it will be important to understand the underlying mechanisms establishing and maintaining tolerance in greater detail. While the circumstances seem fairly evident in the event of full chimerism, the tolerization of pre-existing recipient T cells in mixed and transient chimeras with an intact T cell repertoire still remains incompletely understood. The group from Stanford conducted mechanistic studies in the murine setting. They found that repeated doses of TLI cause severe DNA damage which drives large numbers of lymphocytes into apoptosis. $CD8^+$ dendritic cells are less sensitive to irradiation and possess specialized receptors (Tim-4, DEC205) that recognize products of dying cells. Activated $CD8^+$dendritic cells activate the immunomodulatory enzyme indoleamine 2,3-dioxygenase and trigger host NKT cells to produce IL-4. Under these conditions, the interaction of IL-4-producing NKT cells with myeloid-derived suppressor cells and Tregs contributes to the induction of tolerance. Myeloid-derived suppressor cells thwart alloreactive T cell responses through the release of Arginase-1 which deprives local T cells from the essential amino acid L-Arginine and increases the production of superoxide (93). Furthermore, myeloid-derived suppressor cells exhibit an increased surface expression of PDL1 and prompt the residual T cell pool including Tregs to express PD1 (94, 95). The interaction of PDL1 with PD1 attenuates effector T cell functions while promoting the induction, maintenance, and function of Tregs (96). Expression of PD1 on Tregs was also linked to enhanced secretion of IL-10 which preserved the allograft and chimerism (97).

The group from Boston started to investigate the mechanisms sustaining tolerance in patients that had lost chimerism. They found that both circulating and intragraft $Foxp3^+$ Tregs were increased in tolerant patients while alloreactive T cells were decreased (98). The increase of peripheral Tregs in the blood resulted from proliferation, thymic emigration, and in one patient from conversion of conventional T cells (99). In order to track alloreactive T cells, they defined a genetic fingerprint of T cells responding to donor cells *in vitro* before transplantation using high-throughput sequencing. Those clones were specifically reduced in tolerant patients but remained unchanged in stable kidney recipients on immunosuppressive therapy (100). Since these patients exhibit transient chimerism, it is likely that donor-reactive T cells were deleted by peripheral mechanisms.

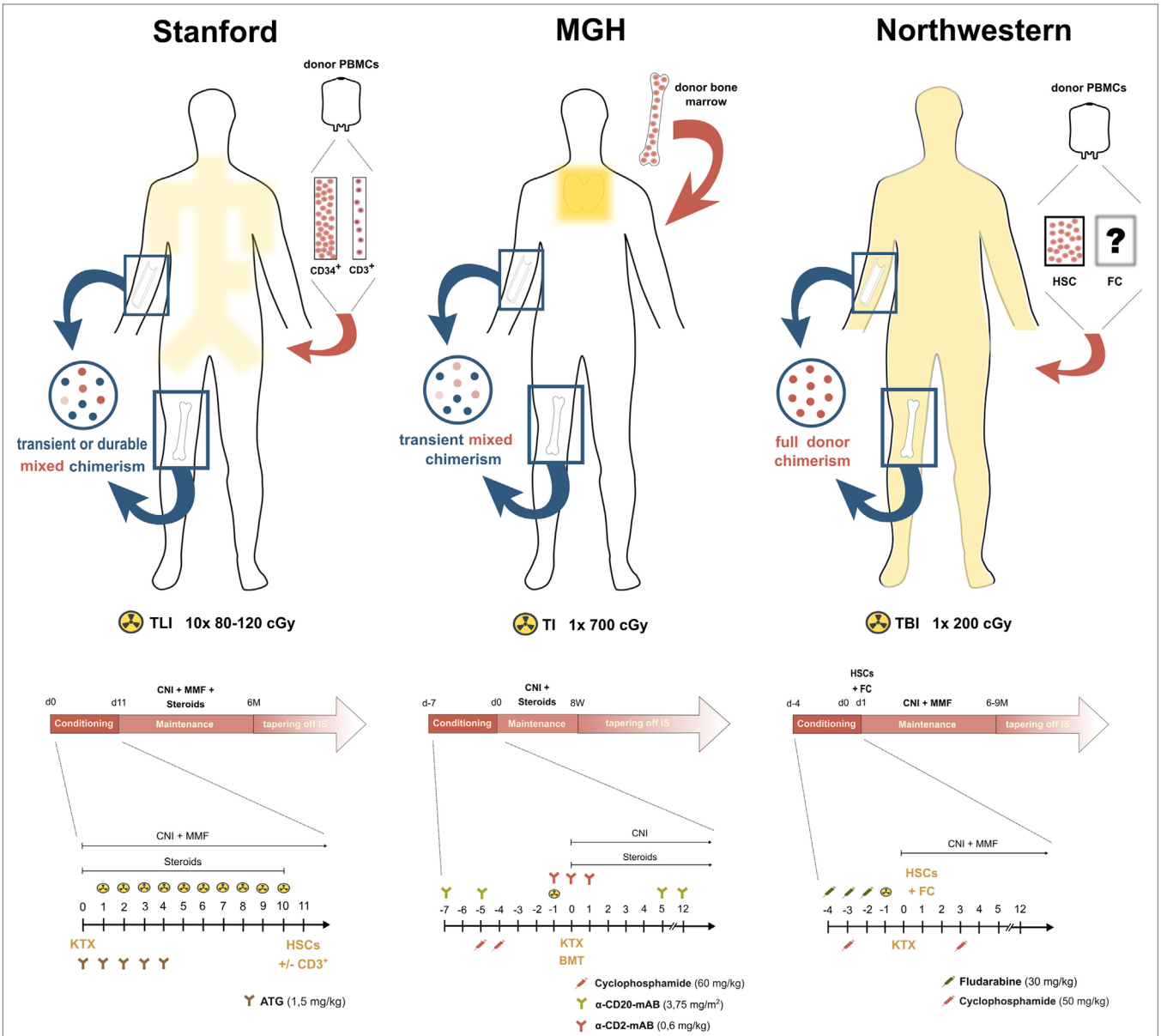

FIGURE 1 | Conditioning strategies in the clinical pilot trials of chimerism-based kidney transplant tolerance. Yellow areas within the silhouettes indicate irradiation fields, the blue circles on the left side display the form of chimerism achieved with each conditioning regimen and the illustration on the right side outlines the administered stem cell product. Below each illustration, an overview about the immunosuppressive course of the respective center is depicted.

Recently, the group exploited a cohort of tolerant non-human primates that had accumulated over the years to investigate the underlying tolerance mechanisms emerging through transient chimerism in more detail. Tolerant recipients lost anti-donor CD8 T cell response while a considerable number of CD4 T cells proliferated against donor cells *in vitro*. A substantial fraction of these responding CD4 T cells constituted Foxp3 Tregs which were induced from conventional T cells in a TGF-β-dependent manner. The suppressive function of these induced Tregs was contact dependent and inhibiting their induction through TGF-β blockade restored CD8 T cell proliferation *in vitro* (101).

FUTURE PROSPECTS

As different as the individual approaches may be, they all agree on the pivotal role of Tregs, suggesting that steering the differentiation of naïve CD4 T cells toward regulation is a critical step for the induction of tolerance. The differentiation of naïve CD4 T cells into various T helper subsets is guided by the surrounding cytokine milieu during T cell receptor stimulation. If CD4 T cells commit to a distinct helper lineage, they start to express certain transcription factors that determine their subsequent mode of action such as immunity, tolerance, autoimmunity, or allergy. Therefore, the specific blockade of immunogenic cytokines could

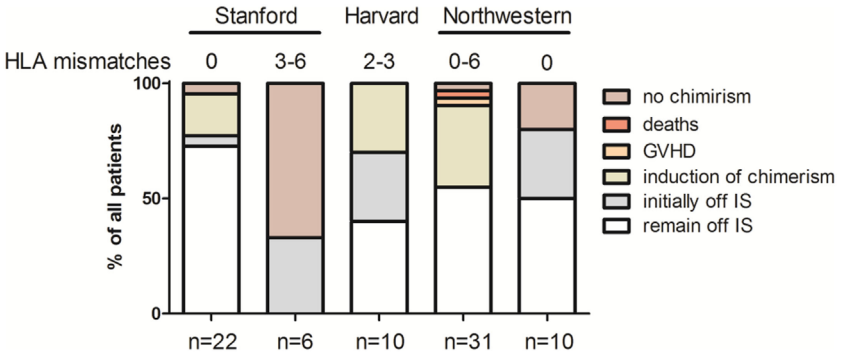

FIGURE 2 | Outcome overview of clinical pilot trials of chimerism-based tolerance in kidney transplantation—graphic illustrations summarizing the key outcomes of the first pilot trials combining hematopoietic stem cell transplantation and kidney transplantation for the purpose of mixed chimerism and tolerance.

TABLE 1 | Key parameters of pilot trials combining hematopoietic stem cell transplantation and kidney transplantation for the purpose of mixed chimerism and tolerance.

Human leukocyte antigen-matching	Boston		Northwestern		Stanford		
	Haploidentical	Haploidentical	Mismatched	Matched	Mismatched	Haploidentical	Matched
Number of mismatches	2–3	2–3	0–6	0	3–6	1–3	0
Patients included	*n* = 10	*n* = 2	*n* = 31	*n* = 10	*n* = 6	*n* = 6	*n* = 22
Induction of chimerism	10/10	2	30/31	8/10	2/6	5/10	21/22
Transient mixed chimerism	10	Ongoing	5	8/10	2	3	9
Stable mixed chimerism	0	Ongoing	3	0	0	2	7
Full donor chimerism	0	Ongoing	16	0	0	0	0
Initially off immunosuppression	8/10	1[a]/2	19/31	8/10	2/6	2/6	17/22
Rejection[b]	3	0	0	3	2	0	0
Graft Loss	3	0	2	0	0	0	0
Remaining off IS	4/10	1[a]/2	19/31	5/10	0	0[a]	16[a]/22
Graft-vs.-host disease	0	0	2	0	0	0	0
Death	0	0	1	0	0	0	0

[a]*Ongoing trial.*
[b]*Only patients where IS was withdrawn are included.*

be envisioned as suitable approach to drive naïve CD4 T cells into the corner of regulation. This strategy has already been proven successful for the treatment of several inflammatory diseases (102) and it will be interesting to see whether this success will continue in the field of transplantation.

IL-6 is probably the most crucial factor guiding the differentiation of naïve CD4 T cells either toward regulation or inflammation. Inhibition of IL-6 signaling with Tocilizumab is already an established treatment option for autoimmune diseases and currently gains traction in the field of transplantation. Coadministration of Tocilizumab to standard GvHD prophylaxis revealed promising potential in a phase I/II trial (32) and is also tested in several transplant trials (103). In the experimental setting, blocking IL-6 displayed a beneficial effect on the induction of chimerism and tolerance in non-human primates and mice (104, 105).

Alternatively, Treg induction could be promoted by establishing the appropriate cytokine environment. The most vital cytokine for the induction and maintenance of Tregs is IL-2. Nonetheless, current maintenance therapies targeting calcineurin inhibit IL-2 secretion and may, therefore, not be suitable for tolerance-inducing strategies (106). The addition of IL-2 has recently been shown to restore the survival and function of Tregs under calcineurin inhibition and to improve allograft survival (107). Harnessing IL-2 to tip the balance between immunity and regulation is, however, a delicate issue as it is essential for both. The compilation and tissue distribution of the IL-2 receptor probably accounts for its pleiotropic effects. IL-2 preferentially binds to its high-affinity receptor which is constitutively expressed on Tregs and rapidly upregulated on conventional T cells and NK cells upon activation, while resting NK cells and memory CD8 T cells constitutively carry the low-affinity receptor (108). Low doses of recombinant IL-2 have been shown to promote tolerance to islet (109) and skin allografts (110) while high doses of IL-2 abrogated kidney allograft tolerance in non-human primates (111). IL-2/α-IL2 complexes accelerated bone marrow rejection in mice independent of their dose implying that the effect of IL-2 also varies with the transplanted tissue (112).

REMAINING HURDLES

The first clinical pilot trials underpinned the difficulties to achieve durable mixed chimerism over MHC barriers. This

issue has often been disregarded in the murine setting where the number of memory T cells is rather low (5–10% of all T cells). Murine models can be improved by enriching recipients with alloantigen primed memory T cells (113) or by using old mice with an increased pool of memory T cells (114). In the human setting, the amount of memory T cells can compromise up to 50% of all circulating T cells (115) from which 1–10% can recognize intact allogeneic MHC molecules through direct allorecognition (116), even in "non-sensitized" recipients through the mechanism of heterologous immunity. Memory T cells are a robust hurdle for tolerance induction due to their lower activation threshold, vigorous effector functions, and their resistance to common immunosuppressive drugs. The presence of memory T cells pre-transplantation has been associated with an increased risk for acute rejection of kidney transplants (117). Pre-exisitng alloreactive memory T cells are most likely generated through recognition of commensal bacteria or environmental antigens (heterologous immunity). Apart from this, memory T cells can occur during homeostatic proliferation following cell depletion or in lymphopenic hosts. Homeostatic proliferation and heterologous immunity have both been shown to impede tolerance induction in mice (118, 119).

Cell-depleting agents enrich memory T cells as they preferentially affect naïve and regulatory T cells (120). T cells with effector/memory phenotype are detectable after Alemtuzumab or ATG induction therapy (120, 121). Moreover, it should be kept in mind that innate immunity which is responsible for the clearance of antibody-coated cells becomes activated (122). Therefore, it would be preferable to block alloreactive cells rather than depleting them. So far CTLA4-Ig (belatacept) is the only costimulation blocker available for clinical use. Besides its availability, CTLA4-Ig also caught attention through its ability to promote chimerism and tolerance in mice (82) and non-human primates (83). Unfortunately, also modern blocking agents have difficulties to keep tab on memory T cells. Terminally effector memory CD4 and CD8 T cells lose CD28 expression and, thus, become resistant to costimulation blockade through CTLA4-Ig (123). Terminal effector T cells are resistant to costimulation blockade even before they lose CD28 expression and their pretransplant frequency has recently been shown to predict episodes of allograft rejection in belatacept-treated patients (124, 125). Increased numbers of $CD28^-$ CD4 and CD8 memory T cells have been associated with a poor outcome in renal transplant recipients (126). IL-15 could restore the proliferation of alloreactive $CD28^-$ memory CD8 T cells *in vitro* (127). Accordingly, strategies are warranted to block IL-15 signaling under costimulation blockade. Blocking the β-chain (CD122) of the IL-15 receptor has recently been shown to synergize with CTLA4-Ig to prolong allograft survival (128).

Alternative strategies include the use of Alefacept which is a fusion protein consisting of extracellular LFA3 domain and human IgG1. It interacts with CD2 which is upregulated on $CD45RO^+$ effector/memory T cells. In this way, Alefacept preferentially targets memory T cells but spares the remaining T cell pool (129). Pre-transplant Alefacept synergized with CTLA4-Ig by targeting $CD8^+$ $CD28^-$ effector/memory T cells (130). Moreover, blocking the integrins LFA-1 and VLA-4 prolonged skin allograft survival in a mouse model of costimulation blockade-resistant rejection mediated by memory CD8 T cells (113, 131).

TOLERANCE OR NO TOLERANCE THAT IS THE QUESTION

One of the fundamental challenges of tolerance-inducing protocols is to identify those patients in which the protocol was successful and which can, thus, safely be weaned off immunosuppression. Stable multi-lineage chimerism has long been regarded as the most robust predictor for tolerance and donor T cell engraftment in particular as a critical parameter (132). However, the prediction of tolerance becomes more sophisticated in the absence of durable chimerism. Accordingly, there is a high demand for other markers that reliably identify tolerant patients.

The optimal biomarker should allow repeated and non-traumatic measurements that faithfully reflect intragraft processes in real-time with a high precision at affordable prices. The demands are certainly high but advancing biotechnological methods offer unknown opportunities. In the effort to find a reliable biomarker for tolerance, conventional transplant recipients retaining normal kidney function over an extended period of time after withdrawal of all immunosuppressive medication (operationally tolerant) have been compared to healthy subjects or immunosuppressed kidney recipients. Tolerant patients exhibited a specific increase in blood $CD4^+$ $CD45RA^-$ $Foxp3^{high}$ memory Tregs in comparison to stable kidney recipients and healthy controls. These Tregs exhibited an increased surface expression of GITR and CD39 together with a decreased demethylation of the Treg-specific demethylated region (133). Since only a minor fraction of the whole Treg pool circulates within the blood, the accumulation of Tregs within the graft drew more interest. Indeed, operationally tolerant patients exhibited an increased proportion of Foxp3 Tregs inside their allografts (134). Besides, dissecting the non-regulatory T cell compartment emerged as useful tool to assess alloreactivity. Analyzing the recipients T cell receptor repertoire by high-throughput sequencing becomes increasing popular on that account. Once a genetic fingerprint of donor-reactive T cell clones has been defined, they can easily be tracked over time. The group from Boston could confirm that a reduction of the alloreactive T cell receptor repertoire correlates with tolerance while an increase is associated with rejection (135).

Whole genome microarray-based transcriptional profiling studies additionally revealed B cells as important markers of kidney tolerance (136, 137). These studies led to the disclosure of a distinct B cell signature in operationally tolerant patients and in patients rendered tolerant through hematopoietic stem cell transplantation (138). This specific B cell profile has recently been deployed to identify a cohort of immunosuppressed patients with improved renal allograft function (139). The circumstance that the B cell signature of tolerant patients was conspicuously similar to those of healthy controls raised the question whether these profiles would merely reflect the absence of immunosuppression. Re-evaluation of the data set revealed that common immunosuppressants bias the expression of the investigated

genes. Nonetheless, a new gene expression profile could be created that was independent of drug-effects (140). In an alternative approach, tolerant patients could be differentiated from stable patients on the basis of elevated miR-142-3p levels in peripheral blood mononuclear cells. Besides, miR-142-3p expression was stable over time and not affected by immunosuppression (141). The fact that miR-142-3p mainly originated from B cells further substantiated their contribution for tolerance.

In line with this B cell profile, tolerant patients also displayed an increased proportion of naïve ($CD20^+$ $CD24^{low}$ $CD38^{low}$) and transitional B cells ($CD20^+$ $CD24^{high}$ $CD38^{high}$) in comparison to immunosuppressed kidney recipients (138). In this respect again, it could not be excluded that the redistribution of B cell subsets might result from the absence of immunosuppression. Patients on azathioprine or prednisone namely feature lower numbers of transitional B cells in comparison to patients off drugs (140). On the other hand, the lack of transitional B cells was associated with kidney allograft rejection (142).

No biomarker has emerged yet that has been sufficiently validated to be used for clinical decision making in the routine setting, but progress achieved so far is encouraging and will likely yield success in the future.

CONCLUSION

After an extended period of hibernation, the dream of tolerance is gaining increasing attention again. The Holy Grail seems to be in one's reach but "he who finds the Grail must face the final challenges—three devices of such lethal cunning" (143). In the event of transplantation tolerance, these final challenges would be to reduce host toxicity, to avoid GvHD, and to improve clinical efficacy. Improving tolerance-inducing strategies is a cumbersome and tedious process but the increasing number of patients undergoing such protocols shows that we are heading into the right direction. Besides, novel approaches such as costimulation blockade, Treg therapy, pro-apoptotic molecules, or immunomodulatory antibodies hold out encouraging prospects for the future. Further important steps toward clinical applicability will be to find appropriate biomarkers that reliably predict tolerance.

On the shady side, the burning desire for eternal organ life sometimes makes us forget the involved risks as can be seen by the two recent cases of GvHD. As Goetz von Berlichingen already said in Goethe's drama: "Where there is bright light, there is also deep shadow." Throughout the course of history progress has often claimed victims but it remains questionable whether this can be justified for the field of (living donation kidney) transplantation. Be that as it may, the rapidly advancing field of immunology will hopefully allow us to achieve tolerance by safer means. In this regard, the field of tumor immunology emerged as major ally. The mechanisms of how tumors evade host immunity provide vital insights for the design of innovative strategies and could guide future directions (144).

Developing tolerance-inducing strategies has always been and continues to be a highly elaborate process. Therefore, it still remains difficult to assess when tolerance-inducing protocols will reach clinical maturity but there is hope that they will not remain a laboratory solution forever.

AUTHOR CONTRIBUTIONS

BM, NG and TW wrote the article and MM revised the manuscript. BM and MM designed the figures and the table.

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REVIEW
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Lactococcus lactis As a Versatile Vehicle for Tolerogenic Immunotherapy

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Genetically modified *Lactococcus lactis* bacteria have been engineered as a tool to deliver bioactive proteins to mucosal tissues as a means to exert both local and systemic effects. They have an excellent safety profile, the result of years of human consumption in the food industry, as well as a lack of toxicity and immunogenicity. Also, containment strategies have been developed to promote further application as clinical protein-based therapeutics. Here, we review technological advancements made to enhanced the potential of *L. lactis* as live biofactories and discuss some examples of tolerogenic immunotherapies mediated by mucosal drug delivery *via L. lactis.* Additionally, we highlight their use to induce mucosal tolerance by targeted autoantigen delivery to the intestine as an approach to reverse autoimmune type 1 diabetes.

Keywords: *Lactococcus lactis*, mucosal tolerance, immunotherapy, autoimmunity, type 1 diabetes

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INTRODUCTION

The mucosal immune system in close interaction with symbiotic bacteria is constantly working to maintain a homeostatic balance between immune activation, which is necessary against vast amounts of exogenous antigens and noxious stimuli, and immune tolerance toward harmless inhaled or ingested antigens and the host microbiota. Disruption of the mucosal homeostasis can result in inflammatory bowel diseases (IBDs) represented by ulcerative colitis (UC) and Crohn's disease (CD). Mucosal tolerance, the ability of mucosally administered antigen(s) to regulate local and systemic immune responses has led to new therapeutic approaches to prevent or treat allergies and autoimmune diseases, such as multiple sclerosis (MS), arthritis, uveitis and type 1 diabetes (T1D). In this regard, mucosal (i.e., nasal or oral) drug delivery is generally the preferred treatment route, as it is easy and provides more patient comfort. Moreover, targeting therapeutics to the mucosal

Abbreviations: APC, antigen-presenting cell; CAT, catalase; CD, Crohn's disease; CIN3, cervical intraepithelial neoplasia grade 3; DC, dendritic cell; DSS, dextran sulfate sodium; FcR, Fc receptor; FDA, Food and Drug Administration; GAD65, glutamic-acid decarboxylase of 65 kDa; GALT, gut-associated lymphoid tissue; GIT, gastrointestinal tract; GM, genetically modified; GPX, glutathione peroxidase; GRAS, generally regarded as safe; GWAS, genome-wide association studies; HPV, human papilloma virus; HSP, heat-shock protein; IA-2, tyrosine phosphatase-like protein ICA152; IAA, insulin autoantibody; IBD, inflammatory bowel disease; IE, intestinal epithelia; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; IFN, interferon; Ig, immunoglobulin; IL, interleukin; iTreg, induced regulatory T cell; *L. lactis*/LL, *Lactococcus lactis*; LAB, lactic-acid bacteria; LP, lamina propria; mAb, monoclonal antibody; MS, multiple sclerosis; NICE, Nisin-controlled gene expression; NOD, non-obese diabetic; OM, oral mucositis; OVA, ovalbumin; PAMP, pathogen-associated molecular patterns; PINS, proinsulin; PTM, posttranslational modification; SOD, superoxide dismutase; SP, signal peptide; T1D, type 1 diabetes; TCR, T-cell receptor; tDC, tolerogenic dendritic cell; TFF, trefoil factors; TGF, transforming growth factor; Th, helper T cell; TLR, toll-like receptor; TNBS, trinitrobenzenesulfonic acid; TNF, tumor necrosis factor; Treg, regulatory T cell; UC, ulcerative colitis; ZnT8, zinc transporter 8.

surfaces may also display superior efficacy over classic systemic immunotherapies. In this review, the biotechnological potential of genetically modified (GM) *Lactococcus lactis* bacteria for mucosal delivery of biotherapeutics as a means to restore local inflammation and (re-)introduce antigen-specific systemic tolerance will be discussed. The abundant successful preclinical applications of these recombinant *L. lactis* harbor great therapeutic potential and will be covered in detail. In addition, we will provide an overview of therapies using GM *L. lactis* that have been tested in clinical trials and discuss how they can be improved.

EXPLOITING MUCOSAL TOLERANCE MECHANISMS

Mucosal tolerance is the active process involving inhibition of antigen-specific immune responses introduced to the organism *via* the mucosal surfaces as found in the lungs and gastrointestinal tract (GIT). It results in suppression of immunological responses to innocuous antigens and avoids unwarranted pro-inflammatory immune responses (1). In healthy individuals, the gut-associated lymphoid tissue (GALT) will only mount an inflammatory response to danger signals, such as toll-like receptor (TLR) activation, when necessary.

The mechanisms of mucosal tolerance are still not completely elucidated, but it is generally accepted that clonal anergy or deletion of reactive cells and induction of regulatory T cells (Tregs) are the two main effector mechanisms (1, 2). High doses of antigen favor clonal anergy or deletion of reactive cells (3). Anergic T cells form defective immunologic synapses with antigen-presenting cells (APCs) resulting in a hyporesponsive state (4). These cells lose their migratory ability and remain at the site of induction where they display immunosuppressive effects on other T cells in an antigen-independent manner (5). Low-dose oral tolerance favors the induction of Tregs. Mucosal tolerance can be induced in the absence of natural Tregs and is established by *de novo* induction of antigen-specific $CD4^+CD25^+Foxp3^+$ Tregs in a transforming growth factor (TGF)-β-dependent manner (6). The current view indicates that intestinally induced Tregs (iTregs) result from an interaction with $CD103^+$ dendritic cells (DCs). After antigen uptake, these $CD103^+$ DCs migrate to the mesenteric lymph nodes where they induce $Foxp3^+$ Treg conversion in the presence of retinoic acid, necessary for expression of two gut-homing molecules (CCR9 and integrin α4β7) (7, 8). Gut-homing iTregs return to the lamina propria (LP) where they expand and are instructed by $CX3CR1^+$ macrophages to produce IL-10, after which they enter the bloodstream to exert systemic effects (9). Tregs can actively suppress autoreactive T cells in a one-on-one manner; however, they also induce antigen-non-specific immune suppression through "bystander suppression" by secreting anti-inflammatory cytokines (10). Autoreactive T cells that respond to a different antigen than that was mucosally given will therefore also be inhibited. Bystander suppression is useful in diseases with unknown autoantigens, multiple autoantigens, or when there is excess inflammation but no autoantigen (11, 12). It is clear that Tregs are critical for continued immune tolerance in the GIT through active control of innate and adaptive immune responses. Dynamic adaptation of Treg populations to the intestinal tissue microenvironment is key in this process.

Although mucosal tolerance happens throughout the entire lifespan, translating this naturally occurring phenomenon into a therapeutic strategy is not self-evident. Many factors need to be taken into account including antigen choice, dose, route, formulation, timing, and frequency of administration. Inducing therapeutic mucosal tolerance by feeding or inhalation of raw protein is a cumbersome task limited by enzymatic degradation in the GIT or nasal secretions, short half-life due to metabolism, limited bioavailability due to molecular size, loss of tertiary structures or posttranslational modifications (PTMs) necessary for antigen recognition, and finally the high cost of development (13). Bringing protein synthesis to the site of tolerance induction would circumvent these technical obstacles. Many researchers have modified the probiotic *L. lactis* to deliver intact therapeutic bioactive proteins to the GIT. This bacterial strain offers several technical advantages and has been tested in diverse applications.

L. LACTIS AS NEXT-GENERATION BIOFACTORIES

Rational for Choosing *L. lactis*

Non-pathogenic lactic-acid bacteria (LAB), such as particular species of lactococci and lactobacilli, have been handled for centuries in the fermentation and preservation of food. Sequencing of the entire genome of a number of heterofermentative *L. lactis* strains (14, 15) has led to the design of a plethora of genetic tools to engineer these gram-positive bacteria into next-generation mucosal delivery tools for bioactive peptides. Moreover, *L. lactis* strains are specifically important because of their use in the production of probiotic dairy products (16). *L. lactis* consists of three subspecies: *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *hordniae*, and *L. lactis* subsp *lactis*. The *L. lactis* subsp. *cremoris* MG1363 is the international archetype for LAB genetics; it is a plasmid-free and phage-cured derivative of the dairy starter strain NCDO712-lacking extracellular proteases. The removal of the pLP712 plasmid, which encodes the *lac* operon and proteases necessary for casein degradation, precludes growth in milk thus limiting propagation of this strain outside well-controlled environmental niches (17). Today, there is sufficient knowledge to support the exploitation of GM LAB, and especially the *L. lactis* subsp. *cremoris*, to distribute therapeutic proteins to the mucosal surfaces (18). The widespread historical use of the *L. lactis* strains in the food industry rendered them with an important "generally regarded as safe (GRAS)" status by the Food and Drug Administration (FDA). Moreover, *L. lactis* strains do not colonize the GIT of humans and animals.

Versatile Protein Delivery Systems

Engineering these bacteria to secrete active therapeutic biologicals can be advantageous for multiple reasons (**Figure 1**):

(1) The *L. lactis*-based delivery system can circumvent the use of large amounts of crude proteins that for a large part will be broken down by digestive enzymes. Furthermore, soluble

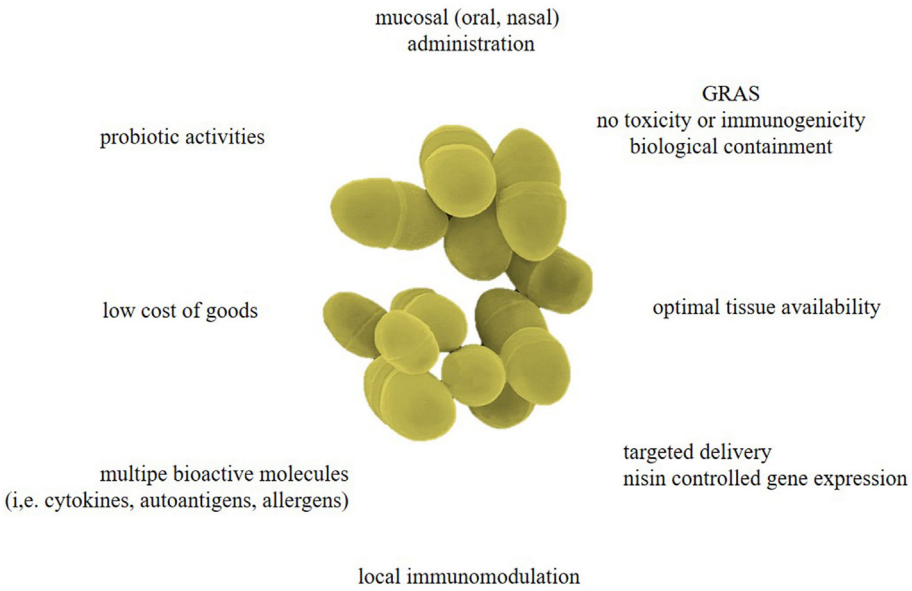

FIGURE 1 | Advantages of *Lactococcus lactis* as designer probiotics for various biopharmaceuticals. As the effectiveness of mucosal-administered antigens diminishes during passage through the gastrointestinal tract (GIT), *L. lactis*-mediated drug delivery could be a safe and low-cost approach for targeted delivery of several bioactive molecules (i.e., cytokines, hormones, antibody fragments, allergens, antigens, etc.), therefore bypassing the adverse effects associated with systemic drug administration. Controlled expression of biopharmaceuticals can offer local immunomodulation and reverse allergy or autoimmunity. These living biofactories have a generally regarded as safe (GRAS) status and can be biologically contained so they cannot colonize the mucosa of the oral cavity or GIT. Moreover, these designer probiotics could reinforce normal immunity to defend the host from infections, inflammatory diseases, and autoimmune responses.

proteins have low immunogenicity and stability when given mucosally (i.e., oral, nasal…). The *L. lactis* can survive the entire GIT while expressing one or more bioactive proteins. This is not only cost-effective but eliminates the variation of how much is digested during transit before reaching its target. Since systemic exposure to the therapeutic biological is negligible, the chance of side effects will also be significantly lower. Although reduced viability in the human GIT due to acid sensitivity is an inherent feature of *L. lactis*, this can be limited through proper enteric coating of freeze-dried *L. lactis* (19). Another modification that can offer robust protection against bile-toxicity and gastric-acid assault is intracellular accumulation of trehalose, a known cryoprotectant, by introducing trehalose synthesizing genes (20).

(2) The *L. lactis* strains can be tailored to express heterologous proteins, either constitutive or inducible, depending on the biological need. Strong constitutive promoters for *L. lactis* have been described (21); however, continuous high-level secretion of specific proteins, such as cytokines and interleukins (IL), could induce intracellular accumulation or degradation, potentially leading to cellular toxicity. Therefore, inducible protein expression may be desired when it comes to regulated drug administration. The most successful inducible expression system is the nisin-controlled gene expression (NICE) system where gene expression is proportional to the concentration of the antimicrobial peptide nisin (22). With this flexible system, the level of gene expression can be restrained by the amount of nisin used for the induction and can be upregulated more than 1,000-fold. Other inducible systems also exist, relying on lactose availability, glucose, pH decrease, or zinc starvation (23). As mentioned, inducible gene expression allows control of therapeutic drug delivery; however, additional genetic engineering may hamper clinical use of the *L. lactis* strains. Steidler and colleagues designed the first elegant biological containment system allowing constitutive gene expression as bacterial viability depends on addition of thymidine or thymine, which is not present in the environment and at meagre amounts *in vivo* (24). Briefly, the thymidylate synthase gene *thyA*, which is essential for DNA metabolism, was replaced with a synthetic human gene, in this case human IL-10. This system combines passive containment, as growth is dependent on supplementation of the essential metabolite, i.e., thymidine, with that of active containment, since thymine-less death is bactericidal rather than bacteriostatic as is the case for most other auxotrophies. This system has many advantages including bypassing the use of antibiotic resistance markers which can disseminate to other microbiota.

(3) Secretion of active proteins is often preferred, as this provides the most straight forward interaction with the mucosa. In theory however, the preferred protein can be produced in the cytoplasm, culminate in the cell membrane, or be distributed from the cells to appear in the environment (secreted) or become anchored at the bacterial cellular surface. Different expression vectors such as pCYT, pSEC, and pCWA have been established, to permit protein targeting

to be either intracellular, extracellular (secreted form), or cell wall-anchored, respectively (18). When using the pCYT vector, the protein is produced but resides in the bacterial cell in the absence of a signal sequence. As such, this approach protects the protein from degradation but depends on cellular lysis to bring it in the extracellular space. The pCYT and pSEC vectors, in which a nisin-inducible promoter controls expression, should be used in the *L. lactis* NZ9000 strain bearing a *nisR,K* chromosomal cassette, required for nisin signaling. Of interest, *L. lactis* strains have a monolayer cell wall which permits direct extracellular secretion. This method allows immediate contact of the active protein with the mucosa but leaves it more sensitive to gastric digestion and proteolysis. The *L. lactis* strains used for recombinant protein expression only have one extracellular housekeeping protease, high-temperature requirement A (HtrA), keeping the effects of proteolysis to a bare minimum. Secreted proteins need an N-terminal signal peptide (SP) and most often the SP of Usp45, the major extracellular protein of the *L. lactis* bacteria, is used (25). However, this SP does not guarantee efficient secretion and other steps, such as protein trimming, may be required to allow successful expression (26). Protein size, the nature of the SP, and the presence of a pro-peptide are important parameters that may hamper protein secretion. Noteworthy, proteins with molecular mass ranging from <10 to >160 kDa have been efficaciously produced in the *L. lactis* strain. This implies that protein size is not a major problem for heterologous protein production in *L. lactis*. On the other hand, protein conformation may be a serious bottleneck for heterologous secretion in *L. lactis*. Several publications indicate that conformation change is the major criterion involved in the stabilization of the precursors and the higher yields measured (27–30). It is also possible to display proteins on the bacterial cell wall by several different anchoring methods, each leading to unique host responses as the proteins will be displayed and exposed to gastric degradation differently. The recombinant molecule can be attached to the membrane layer using a transmembrane anchor or a lipoprotein-anchor, or to the cell wall by a covalent link using sortase-mediated anchoring *via* the LPXTG motif (31). The target protein can be synthesized by the *L. lactis*, however, it is also possible to anchor recombinant proteins made in different expression strains by non-covalent binding-domain-mediated anchoring (31–33). This can be extremely useful for delivering proteins that can only be expressed by bacterial strains that are not suited for clinical practice or if PTMs are required that can only be done by eukaryotic cells. The most prevalent PTMs include glycosylation, methionine oxidation, asparagine and glutamine deamidation, and proteolysis. These PTMs not only represent obstacles for precise and reliable bioprocessing but also they may be necessary to induce the appropriate immune responses. The discovery of the *in-trans* surface display system has opened the way to facilitate glycoprotein delivery. This strategy was utilized to produce the tyrosinase related protein-2 (TRP-2-cA) glycoprotein fused with the *L. lactis* N-acetylmuramidase C-terminal LysM cell wall anchor, cA, in mammalian Chinese Hamster Ovary (CHO) cells before subsequent binding to *L. lactis* cell wall (34). *L. lactis*-based secretion of deamidated peptides has also been described. Here, we present the example in which two glutamine residues within the α-gliadin peptide were changed into glutamic acids to stimulate the deamidated immunodominant α-gliadin response for HLA-DQ8 carrying celiac disease patients (35).

Lactic-acid bacteria displaying antigens on their cellular surface have been shown to be able to elicit strong immune responses with one of the most detailed examples being mucosal vaccination against the human papilloma virus (HPV) oncoprotein E7. Mice vaccinated with *L. lactis* expressing the HPV E7 antigen and IL-12 were protected against HPV-16-induced tumors (36). Interestingly, they tested three cellular locations for the oncoprotein expression in *L. lactis* and found that intracellular production ultimately lead to protein degradation and the cell wall-anchored form of E7 gave the strongest immune response (37, 38). A similar therapy, using attenuated *Lactobacillus casei*, was evaluated for safety and efficacy in patients with cervical intraepithelial neoplasia grade 3 (CIN3) as a result of HPV. No adverse events were reported, demonstrating the safety profile of these GM LAB, and oral vaccination induced successful anti-neoplasm mucosal immunity (39). The authors hypothesized that the bacterial cell wall, more specifically the proteoglycan compounds, may display adjuvant properties, thus enhancing the immune response (40). It is possible to increase interactions with host cells by co-expression of adhesive factors on the bacteria cell wall as an adjuvant.

While this way of protein expression opens doors to new approaches, it remains difficult to predict which route is better. An important factor is the amount of protein expressed and how relevant that is when comparing secreted proteins to cell wall-anchored proteins. In the case of bioactive proteins, such as cytokines and hormones, extracellular production will be critical in order to get a functional molecule. Ultimately, it will be necessary to study each protein on a case-by-case basis.

Probiotic Properties

Probiotic activities (i.e., having health-promoting properties) have been mostly studied in other LAB, such as those of the *Lactobacillus* genus (41). In a recent randomized double-blind placebo trial in human subjects, the probiotic properties of three *Lactobacillus plantarum* strains were studied. All strains influenced the intrinsic repair processes of the small intestinal mucosa on a gene transcriptional level and the strongest effects were demonstrated by *Lactobacillus plantarum* TIFN101 (42). The ability to adhere to the intestinal mucosa is one of the more important selection criteria for probiotics as adhesion is considered to be a necessity for colonization. The *Lactococcus* genus has often been overlooked because it is not part of the normal microflora and it does not proliferate or colonize at a specific location. Nonetheless, there are some studies attributing beneficial anti-inflammatory effects to certain subspecies of *L. lactis* (**Figure 1**). For example, the *L. lactis* subsp. *lactis* NCDO 2118 reduces symptoms of recurrent colitis in the dextran sulfate sodium (DSS)-induced colitis

model. Early IL-6 production may enhance mucosal repair and preserved colonic IL-10 production which could be responsible for the marked anti-inflammatory effects (43). Furthermore, oral treatment with NCDO 2118 nearly doubled the frequencies of $CD4^+CD25^+LAP^+$ Tregs in mesenteric draining lymph nodes and spleen. Another study found that oral administration of milk fermented with *L. lactis* subsp. *cremoris* FC protected host animals against influenza virus infection (44). The protective effects against influenza virus were mostly derived from the cell components of *L. lactis* subsp. *cremoris* FC and its metabolites, such as exopolysaccharide. Many studies have shown that *L. lactis* can support barrier function in terms of improved mucus, production of antimicrobial peptides, and secretion of soluble immunoglobulin (Ig) A (45).

L. LACTIS FOR MUCOSAL DELIVERY OF CYTOKINES AND ANTIGENS

One well-studied application of modified *L. lactis* is its potential for treating pathologies of the mucosal layer. IBD refers to the chronic relapsing inflammatory diseases resulting from a breakdown of tolerance in the GIT, and include CD and UC. Many current treatments of IBD approach the pathology by systemic drug administration. Orally administered *L. lactis* can specifically deliver the drug to the lumen of the gut, allowing topical treatment of the inflicted area.

The Case of the Anti-Inflammatory Cytokine IL-10

One of the first GM *L. lactis* was designed to treat IBD. IL-10 plays a pivotal role in intestinal homeostasis (46) as $IL\text{-}10^{-/-}$ mice spontaneously develop colitis (47) and single-nucleotide polymorphisms in IL-10 signaling have been associated with IBD in genome-wide association studies (GWAS) (48–50). Treatment with parenteral administration of recombinant IL-10 showed some efficacy in human trials for CD; however, full remission was never obtained (51–53). Some discrepancies remain concerning the required dose, since high doses exert immunostimulatory effects and lower doses lack clinical efficacy. An important unanswered question regarding systemic IL-10 administration is whether the cytokine reaches its target as the serum half-life of IL-10 is less than 3 h and IL-10 has limited mucosal bioavailability (54). Oral delivery of the recombinant protein might provide an attractive alternative; however, it is complicated by the extreme acid sensitivity of IL-10 in addition to the general complications of oral protein delivery (i.e., degradation in the GIT, hepatic first-pass metabolism and high-production costs). Intragastric administration of IL-10 secreting *L. lactis* (*LL-IL-10)* circumvents many of these issues, bringing the cytokine synthesis directly to the inflamed mucosal tissues. Pioneering work by Steidler et al. showed that with *LL-IL-10* much lower amounts of IL-10 were required to reduce inflammation in DSS-induced chronic colitis, compared with systemic recombinant IL-10. Furthermore, it was possible to prevent disease-onset in $IL\text{-}10^{-/-}$ mice (55). One of the many anti-inflammatory effects of IL-10 is the generation of tolerogenic dendritic cells (tDCs), which regulate intestinal homeostasis by inducing suppressor T cells. *LL-IL-10* can modulate immature DCs *in vitro* to upregulate CD83 and CD86, which in turn will induce suppressive helper T cells (Th). This suppressive effect was 40 times more efficient with *LL-IL-10* than exogenous recombinant human IL-10 (56). Even in an *in vitro* setting, where a potentially hostile GI environment is eliminated, it is still more efficient to deliver IL-10 by the *L. lactis* delivery system rather than as a soluble protein.

Based on these results, a small safety and tolerability phase-I clinical trial in 10 CD patients was initiated. Participants were treated with *L. lactis* in which the *thyA* gene was replaced with the human IL-10 gene (24). This presented the first safety test in humans for this biological containment system. Treatment with *LL-IL-10* showed to be harmless, well tolerated and effectively contained *in vivo*, encouraging further use for human application (57). A decrease in disease activity was also observed in 8 out of 10 patients which, combined with a promising safety profile, encouraged further testing. In a larger phase-II trial (NCT00729872) the safety and environmental containment were confirmed, which were the primary objectives of the study. However, no statistically significant differences were observed between placebo and *LL-IL-10* treatment in mucosal healing. The lack of clinical effect may have been due to insufficient exposure to viable bacteria which in turn can be improved by several technological modifications (19, 20, 58).

Perhaps this approach was still not sufficient to protect the synthesized IL-10 from gastric acidity and proteases. A novel tactic for IL-10 delivery by *L. lactis* was designed in 2009, where a plasmid, pValac, was constructed for DNA delivery into eukaryotic host cells. This strategy has demonstrated that expression of the protein encoded in the DNA vector was expressed by epithelial cells of the large intestine (59). Delivery of DNA into the target cells allows for appropriate protein expression (i.e., with the necessary PTMs and correct conformational epitopes) and recognition by the host. The *L. lactis* co-expressing fibronectin binding protein A (FnBPA), which likely acts as an adhesin facilitating plasmid internalization, was slightly more successful and was also able to increase intestinal secretory IgA production, an important first-line of defense mechanism. Nonetheless, this IL-10-encoding DNA plasmid pValac has been cloned into several *L. lactis* strains and was consistently able to diminish intestinal inflammation in a chemically induced murine model for colitis (60).

Another approach to enhance enteric IL-10 production is based on the host immune evasion strategy of the enteropathogenic *Yersinia* species. These bacteria can secrete the soluble protein low-calcium response V antigen (LcrV) that stimulates host-IL-10 production which in turn will facilitate bacterial survival through its anti-inflammatory effects, more specifically on IFN-γ (61). Oral administration of *LL-LcrV* significantly enhanced colonic IL-10 production in a TLR-2-dependent manner and was able to prevent and improve colitis in two different mouse models (62).

Since IL-10 is the key regulator of inflammatory cascades, it has also been studied in other inflammatory diseases outside the GIT. Allergic asthma, the inappropriate immune response to harmless proteins or allergens, is a major health problem with increasing prevalence worldwide (63). It is a hallmark Th2 disorder marked

by recruitment of eosinophils, IgE switching, and production of several chemokines that further attract additional leukocytes. Local administration of IL-10 to this respiratory site by means of intranasal administration of *LL-IL-10* was able to modulate acute airway inflammation in a murine model (64). Two xylose-inducible expression systems were tested to target IL-10 to the cytoplasm or extracellular medium (secretion). A decrease of Th2 cytokines (i.e., IL-4, IL-5), a decreased presence of local eosinophils, and improved histology of the lung tissue were observed. Interestingly, these effects were most outspoken with the *L. lactis* strain-producing cytoplasmic IL-10. It is noteworthy that these effects were not mediated by an increase of $CD4^+Foxp3^+$ Tregs. Perhaps increasing *L. lactis* inoculation to more than two exposures could improve therapeutic outcomes and have an effect on Tregs as well.

TNF-α Antagonists

Systemic treatment with tumor necrosis factor (TNF)-α antagonists, blocking its pro-inflammatory effects, has become a standardized treatment for IBD. However, 10–30% of patients are primary non-responders and 23–46% become secondary non-responders over time, in part due to immunogenicity (65). Furthermore, there are serious side effects leading to a low compliance associated with this therapy (66). Again, the rationale here is that systemic administration requires a much higher dose in order to obtain sufficient amounts at the target site, which is the inflamed GIT. The *L. lactis* has been engineered to secrete mono- and bivalent neutralizing anti-TNF-α nanobodies and is able to ameliorate DSS-induced chronic colitis in mice to a comparable degree as the *LL-IL-10* (67). Interestingly, *LL-anti-mTNF*-α also shows some effects on disease severity in established colitis in $IL10^{-/-}$ mice. The anti-inflammatory effect is limited to the intestine, indicating that there is no generalized immune suppression which would leave patients vulnerable to infections or malignancies.

Biologic Therapy with IFN-γ

Interferon (IFN)-γ is first and foremost known as a key pro-inflammatory cytokine produced by T cells and natural killer cells. However, it also exerts anti-inflammatory and immunoregulatory activities making it a complex, though interesting, drug target. Sheikh and colleagues demonstrated that IFN-γ exerts its anti-inflammatory properties through inhibition of IL-23. In germ-free mice colonized with enteric microbiota, inhibition of colonic *Il23a* correlated with IFN-γ generation. Colonic $CD11b^+$ cells seem to be the main source of IL-23 and are an IFN-γ target (68). Microbial production of soluble and functional recombinant IFN-γ was achieved in *Escherichia coli* (69). While many therapeutic applications require large amounts of recombinant IFN-γ for parenteral use, it has been shown that oral IFN-γ also elicits systemic suppressive effects which cannot be inhibited by circulating antibodies against IFNs (70). The production of mature, biologically active murine IFN-γ by *L. lactis* allows both purification and therapeutic use to target IFN-γ to the mucosal immune system (71). This formulation may prove to be useful in cases where IFN-γ has therapeutic effects on mucosal afflictions, such as oral submucous fibrosis (72).

Trefoil Factors Taking the Floor

Another successful therapeutic cloned into the *L. lactis* system are the trefoil factors 1, 2, and 3 (TFF1–3), a family of peptides which can promote epithelial wound healing and protect it from further damage, including the mucous epithelia from the GIT. TFFs may be involved in IBD pathogenesis and are a prospective treatment option. Mice treated with *LL-mTFF-1/2/3* after DSS-induced colitis showed reduced neutrophil activation and reduced epithelial damage. Purified TFF was administered rectally and was also able to slightly improve these parameters; however, doses up to 1,200-fold higher than that secreted by *LL-mTFF* were tested and similar efficacy was still not achieved (73). TFFs have also been explored as therapeutics for the treatment of oral mucositis (OM), a painful, debilitating, and common complication associated with cancer treatment with limited treatment options available (74). A phase-II trial that evaluated the efficacy of recombinant human intestinal trefoil factor (rhITF) oral spray for prevention of OM showed the spray was safe, well tolerated, and effective in reducing the frequency and severity of OM in patients with colorectal cancer treated with chemotherapy (75). Human TFF1 was cloned into the *L. lactis* and clinically formulated into a mouth rinse, coded AG013 (76). Encouraging preclinical data from a hamster model formed the basis for a phase-Ib trial (NCT00938080) with AG013 in patients with locally advanced head and neck cancer receiving induction chemotherapy (77). No AG013 bacteria were detected in blood from any subjects, even those given the highest dose (1.2×10^{12} CFU/day). In addition, live bacterial load and human TFF1 levels in saliva and oral mucosa were not significantly different between different doses (2.0×10^{11}; 6.0×10^{11}; and 1.2×10^{12} CFU/day) (77). Preliminary efficacy analysis showed reduced duration of OM, supporting further study in an ongoing phase-II clinical trial (NCT03234465).

An interesting fact about the abovementioned therapies is that much lower doses of therapeutic biologicals are needed when synthesized by a *L. lactis* strain than when the biological exposed to the GIT as a purified protein. The *L. lactis* are able to make close contact with the immune cells of the LP and the intestinal epithelia (IE), which likely means the drug is made available in extremely close proximity to its target cells. Inflammation-induced architectural changes in the mucosa increase this contact, perhaps explaining the widespread success of the *L. lactis* as a mucosal delivery tool for IBD. Oral formulation of cytokine administration, compared with the conventional parenteral route, in its own may be sufficient motivation to give these bugs a closer look.

Heat-Shock Proteins (HSPs) Revisited

Many treatments with GM *L. lactis* aim to treat the symptoms of excessive inflammation. It is also possible to tackle an underlying pathogenesis of IBD, namely, a breakdown of immune tolerance to microbiota using *L. lactis* (78). In this case, HSPs are extremely interesting antigens to study since they are stress proteins known to be overexpressed in inflamed tissues in IBD (79–81), as well as linkers of pathogen-associated molecular patterns (PAMPs) expressed by commensal bacteria. T-cell-specific inflammatory immune responses to bacterial and human homolog peptides derived from HSP60/65 were found in mucosal biopsies from patients with pediatric CD (82). Circulating IgA antibodies

against mycobacterial HSP65 were also elevated in CD patients (83). Clearly, bacterial and/or self-HSP play a part in the pathogenesis of IBD, which motivated their use in a therapeutic setting. Interestingly, HSP boost and direct potent peripherally induced Tregs toward inflammatory sites to reinstate self-tolerance (84). Oral administration of *L. lactis* secreting HSP65 is able to completely prevent DSS-induced colitis in an IL-10/TLR-2-dependent manner (85). Again, $CD4^+LAP^+$ and conventional $CD4^+Foxp3^+$ Tregs were induced.

Protease Inhibitors

Although many therapies using GM LAB that aim to deliver anti-inflammatory molecules to the intestine showed promising results in murine models for IBD, it is not guaranteed they will also be successful in clinical trials. One technical hurdle might be applicable to all anti-inflammatory cytokines; they must be able to reach the immune cells located in the LP to exert their effects. The DSS-induced colitis model is characterized by severe IE damage. Perhaps in patients, with less severe destruction and exposed mucosal immune system compared with the murine model therapies such as the *LL-IL-10* will not be as efficient. Recent work has shown that high proteolytic activity is found in the intestine of patients with CD and UC. This enhanced proteolytic activity is mostly the result of infiltrating immune cells as well as proteases involved in apoptosis (86). Additionally, there is genetic evidence supporting a role for proteases and protease inhibitors in IBD (87). These observations lead researchers to compare the efficacy of orally administered *L. lactis* secreting the conventional anti-inflammatory cytokines IL-10 and TGF-β with novel strains secreting serine protease inhibitors Elafin and Secretory Leukocyte Protease Inhibitor (SLPI). Interestingly, the GM *L. lactis* secreting Elafin was most successful as it displayed the most significant reduction of inflammation (88). Moreover, endogenous protease inhibitors are also released by the IE, where these therapeutics are delivered, indeed supporting their superior effects compared with IL-10 and TGF-β. Protein quantity, and therefore drug quantity, plays an important role in dose-dependent therapeutics. To address this issue, the authors developed a mutated *L. lactis* strain with an inactivated HtrA protease (htrAΔ). This inactivation led to increased protein production and secretion with only minor effects on bacterial growth. *LL-htrAΔ* secreting Elafin was even more successful in reducing intestinal inflammation, showing that perhaps other therapeutics cloned into a wild-type *L. lactis* might also be more therapeutically effective in a protease-deficient strain.

SOD and CAT Enzymes

Another contributing factor in the pathogenesis of IBD is oxidative stress signaling which leads to the production of reactive oxygen and nitrogen species that have debilitating effects on the mucosal layer and partake in disease initiation (89). While the human body has natural anti-oxidative capacities, these cannot handle the excessive oxidant load leading to oxidative stress. Some endogenous intracellular antioxidant enzymes, such as superoxide dismutases (SODs), catalase (CAT), and gluthathione peroxidase (GPX), could be used therapeutically to decrease the level of gastrointestinal oxidative stress. In fact, when GM *Lactobacillus casei* modified to produce SOD or CAT were given to mice prior to the induction of trinitrobenzenesulfonic acid (TNBS)-induced CD, they recovered faster and showed lower intestinal inflammation than controls (90). The anti-inflammatory activity of a *Lactobacillus gasseri* strain-producing manganese SOD was shown to be associated with a reduction in the severity of colitis in IL-10-deficient mice. Another ingenious method for SOD delivery was discovered by evaluating the beneficial effects of fermented milk products on murine colitis (91) and on human gut homeostasis (92). Host antimicrobial actions result in lysis of the *L. lactis* subsp. *lactis CNCM I-1631* (*L. lactis* I-1631) bacteria and subsequent release of cytoplasmic SOD that scavenges extracytoplasmic reactive oxygen species and results in colitis attenuation (93).

Considering the abovementioned data, along with others reviewed elsewhere (18, 94, 95), we believe that this clearly underlines the strong potential for *L. lactis* as a tool to deliver active therapeutics to the mucosa to elicit robust local immune effects.

L. LACTIS FOR THE GENERATION AND MAINTENANCE OF ANTIGEN-SPECIFIC TOLERANCE

A breakdown of antigen-specific tolerance can lead to numerous disorders, including food allergies and autoimmune diseases like MS, arthritis, and T1D. The *L. lactis* carrier showed to be effective in delivering therapeutics to the mucosa, opening the door to study its potential to reinstate systemic antigen-specific tolerance.

Ovalbumin (OVA) As a Model Antigen

Obtaining antigen-specific tolerance in a therapeutic protocol would be desirable for many diseases which cannot always be prevented. Huibregtse and colleagues showed that it is possible to induce antigen-specific peripheral tolerance by oral administration of *L. lactis* secreting ovalbumin (*LL-OVA)* in OVA-immunized transgenic mice with OVA-specific $CD4^+$ T-cell receptors (TCR) (96). Interestingly, *LL-OVA* was able to induce APC-mediated OVA-specific T-cell proliferation at much lower levels than purified OVA. The precise mechanism by which *L lactis* enhances tolerogenic signals remains unclear. However, *LL-OVA* clearly induced a splenic regulatory $CD4^+CD25^-Foxp3^+$/CTLA-4^+ population, likely iTregs. This confirms the suggestions that *L. lactis* alters DC functions skewing them toward Treg inducers (56). *L. lactis* may directly modulate antigen processing and presentation as well as the expression of co-stimulatory molecules on DCs. Most of the luminal OVA after *LL-OVA* feeding was found in the cecum and colon, and most of the mucosal OVA was found in the terminal ileum. Currently, it is undetermined which intestinal site is most important for tolerance induction. DCs residing throughout the GIT are able to directly sample luminal antigens through the IE. Due to the association with the IE, *L. lactis* may allow more efficient antigen uptake than is available through oral administration of OVA. Finally, antigen-specific IL-10 production was only observed in mice treated with *LL-OVA* (compared with an empty vector control or purified antigen) and OVA-specific suppression was dependent on TGF-β, a hallmark characteristic of Th3 cells. Again the *L. lactis* empty

vector control itself had some effects, being able to significantly reduce the delayed-type hypersensitivity response to OVA as well as moderately decrease OVA-specific $CD4^+$ T-cell proliferation. Collectively, these findings highlight how different Tregs (iTregs and Th3) can overlap in functionality and phenotype and hint at the complexity of the regulatory pathways involved in mucosal (oral) tolerance. Moreover, these promising results were obtained in a TCR-transgenic mouse and are not guaranteed to be replicable in a host with a normal, broad TCR repertoire. Nonetheless, if this tool can induce Tregs that can confer bystander suppression to T cells reactive to unknown or multiple antigens, this approach would be extremely valuable (97).

Food and Inhaled Antigens

Several immune-mediated diseases (such as type-I allergies) are triggered by well-defined antigens. Therefore, tolerance protocols aiming at targeting these food- or inhaled airborne antigens are clearly warranted. It was shown that early feeding or intranasal administration of high antigen doses could induce tolerance in mice and that tolerized mice had more IL-10- and TGF-β-producing T cells in their Peyer patches (1, 98). A valuable asset of *L. lactis* as bacterial delivery vehicles for vaccines is their potential to elicit antigen-specific secretory IgA responses at mucosal surfaces. Intranasal or oral inoculation of mice with *L. lactis* engineered to produce β-lactoglobulin (*LL-BLG*), a major allergen in cow's milk, induced specific anti-BLG fecal IgA antibodies (28). Furthermore, pretreatment with *LL-BLG* in the presence of IL-12-producing *L. lactis (LL-IL-12*) prevented a Th2-type immune response after systemic sensitization with BLG by developing a strong Th1 response that correlated with the amount of recombinant BLG produced (99, 100). Delivery of a deamidated gliadin epitope, an immunodominant epitope in celiac disease, by *L. lactis* to transgenic humanized non-obese diabetic (NOD) AB°DQ8 mice was also able to induce antigen-specific tolerance mediated by $Foxp3^+$ Tregs that function in an IL-10 and TGF-β-dependent mechanism (35). While some probiotic LAB, *Lactobacillus reuteri* and *Lactobacillus casei*, can prime DCs to drive the development of IL-10-producing Tregs, supplying IL-10 to the intestine by delivery using *L. lactis* is also an option (101). Indeed, oral administration of *LL-IL-10* diminished anaphylaxis significantly in an animal model of food allergy. Preventative treatment with *LL-IL-10* inhibits antigen-specific serum IgE and IgG_1 production and increases antigen-specific GI IgA levels (102). Interestingly, some of the immune effects can be attributed to the *L. lactis* since the wild-type control also reduces antigen-specific antibodies and moderately increases IL-10 secreting cells in the Peyer's patches.

With respect to inhaled allergens, the modulation of allergic immune responses to the major dust mite allergen Der p2 by recombinant *L. lactis* has recently been described (103).

Autoantigens: *L. lactis* As a Potential Immunotherapy for Autoimmune Type 1 Diabetes

Type 1 diabetes is a chronic autoimmune disease characterized by immune-mediated destruction of the pancreatic insulin-producing beta cells by autoreactive $CD4^+$ and $CD8^+$ T cells (104). The eventual total loss of insulin production causes patients to become reliable on exogenous insulin to manage their glycemia levels. The prevalence of T1D is estimated to be 20 million patients worldwide, with an alarming increase in incidence rate in children younger than 5 years old (105). Treatment with exogenous insulin is successful in bringing the glycemia to normal levels, both in fasting and postprandial settings. However, vascular complications, both of the macrovascular and microvascular blood vessels, are responsible for the morbidity and mortality of T1D (106). These life-threatening complications and rise in incidence emphasize the need for a cure.

Therapies Broken Down by Disease Stage

Novel immunotherapies aim to restore antigen-specific tolerance without notable immune suppression. Which therapeutic approach is taken depends heavily on the disease stage which correlates with the rate of beta cell decline (107). The aim of *primary prevention* is to prevent islet autoimmunity in genetically susceptible young individuals. *Secondary prevention* protocols aim to prevent autoantibody-positive individuals from progressing to overt dysglycemia. In *tertiary intervention* protocols, the goal is to minimalize further beta cell loss and improve glycemic control after diagnosis (107). Carrying out trials at these different stages each come with their own limitations, such as extensive screening to identify the target population for primary and secondary preventions and limited therapeutic benefit in the case of success for tertiary preventions. Since autoimmunity, marked by the presence of autoantibodies produced by B cells, is present before onset of clinical symptoms this is an appropriate stage of the disease for antigen-specific immune interventions (108). This does not exclude their use in a tertiary intervention stage when combined with islet supplementation to ensure sufficient beta cell mass is present to reach normal glycemic control in the case of successful immune modulation.

Nowadays, the use of anti-CD3 monoclonal antibodies (mAbs) has moved from the bench to the bedside. Initial studies with anti-CD3 mAbs demonstrated that a short-term treatment with a low dose (5 μg/day intravenously for five consecutive days) could induce durable tolerance to beta cell antigens without inducing general immunosuppression in preclinical models (109). Anti-CD3 mAbs did not remove the pancreatic insulitic lesions but were ineffective in prophylactic settings, indicating that the timing of treatment with anti-CD3 is critical for inducing long-term tolerance. Therefore, researchers are currently evaluating the window of opportunity for anti-CD3 therapy, with a phase-II trial (NCT01030861) to evaluate subjects further from the time of initial diagnosis. The first anti-CD3 mAb, OKT3, was used to reduce graft rejection after transplantation. Due to its Fc receptor (FcR) binding properties, it induced a cytokine storm making it unsuitable for clinical use. Two humanized non-mitogenic anti-CD3 mAbs, teplizumab and otelixizumab, were developed for clinical trial testing. Phase-III trials in new-onset T1D patients showed a degree of clinical efficacy demonstrated as better C-peptide response, lower insulin requirements and better glycemic control. However, these mAbs failed to meet

their primary endpoints, such as significant change in clinical outcome (110–113). Based on these observations, it seems that these agents alone do not restore normal glucose control, and future approaches will likely require combinations of agents with complementary immune or metabolic activity.

Targeted versus Ignored Beta Cell Antigens

An important determinant for the success of an antigen-based immune intervention is the choice of antigen. Antigen-specific therapies have mainly concentrated on administering the autoantigens themselves. The most common and abundant autoantigens in T1D patients and high-risk individuals are (pro)-insulin (P)INS, glutamic-acid decarboxylase of 65 kDa (GAD65), tyrosine phosphatase-like protein ICA152 (IA-2), and zinc transporter 8 (ZnT8) (114, 115). These epitopes are the target of autoantibodies and can activate autoreactive $CD4^+$ and $CD8^+$ T cells. Already more than 25 years ago it was shown that oral administration of insulin was effective in delaying the onset and decreasing the incidence of diabetes in NOD mice (116). Since then many variations of insulin administration, as well as several other autoantigens, were tested in animal models with overall very positive results (117). These encouraging results led to numerous clinical trials that all failed to meet their primary endpoints [reviewed in Ref. (107)]. It became clear that successful clinical translation of antigen-specific therapies would rely on a variety of factors, such as antigen selection, antigen dose, antigen bioavailability, route of administration, and timing of intervention (118). Moreover, since the ability of beta cell autoantigens to prime the immune system diminishes with disease development, beta cell antigens that are not uninvolved in the autoimmune process can avoid disease more successfully in NOD mice. Oral delivery of T1D-relevant antigens *via* the *L. lactis*, as a means to circumvent these pharmacokinetic limitations, together with HSP65 was already proven successful in reducing diabetes incidence in NOD mice in an antigen-dependent manner (119, 120).

Over the years, it has become clear that the immunological defects of T1D are complex and to halt or prevent T1D in humans in which T1D pathogenesis appears to be very heterogeneous will require more than one single agent. The T1D community advocates the use of combination immunotherapies targeting multiple biological pathways in a synergistic manner (121). We propose that mucosal administration of T1D-relevant autoantigens in combination with low doses of systemic immune modulators and/or anti-inflammatory agents would be a means to restore long-term antigen-specific tolerance while minimizing the risk of side effects (108, 122).

Mechanism of Action? Biomarkers of Success?

Our group demonstrated that oral administration of *L. lactis* secreting PINS and IL-10 (2×10^9 CFU/day/6 weeks) combined with systemic low-dose anti-CD3 (2.5 μg/day/5days) (combi-PINS therapy), stably reversed new-onset diabetes in around 60% of NOD mice (123, 124). Compared with anti-CD3 monotherapy, combi-PINS reverted diabetes faster and cured mice had more stable glycemia levels during therapy and the follow-up period. This was also shown for oral delivery of *LL-GAD65*$_{370–575}$ + *IL-10* with anti-CD3 (combi-GAD65 therapy) in a similar manner (125). Both combination therapies were well tolerated showing no signs of weight loss or intestinal inflammation. A remarkable observation is that combi-GAD65$_{370–575}$ had a higher efficacy in mice with severe hyperglycemia at diagnosis (>350 mg/dl) than anti-CD3 alone as well as combi-PINS, alluding to the importance of antigen choice. Our data imply that splitting a large autoantigen may expose several cryptic elements and prime more efficiently regulatory responses than the whole autoantigen. Inducing regulation to specific beta cell autoantigenic epitopes may be safe as (1) the regulatory immune responses will be specific and (2) it is less likely to boost autoreactive T-cell responses since cognate T cells are not previously activated.

This therapeutic effect was not accompanied with proliferation of functional beta cells but rather a preservation of beta cell mass and a reduction in severe insulitis. Coadministration of gut-delivered IL-10 *via L. lactis* also improved reversal rates. The importance of gut-specific IL-10 in the balance between the intestinal mucosa and the immune system was demonstrated by the development of transgenic mice (Fabpi-IL-10 mice) that overexpress this cytokine only in their IE (126). Compared with wild-type mice, Fabpi-IL-10 mice had high numbers of intraepithelial lymphocytes (IELs) and IgA-producing B cells in their LP. Activated IELs in Fabpi-IL-10 mice had lower levels of Th1 cytokines TNF-α and IFN-γ but increased levels of the Th2 cytokine TGF-β. These data provide evidence for an *in vivo* lympho-epithelial cross-talk, by which cytokines locally produced by intestinal epithelial cells (IECs) can regulate intestinal immune responses without systemic modifications. Certainly, intestinal Tregs are targeted by the mucosal delivery of IL-10 and it may also directly modulate Th17 cells since these cells also express IL-10 receptors (127).

T-cell responsiveness to disease-unrelated antigens was not altered as unmanipulated NOD mice and combi-PINS-cured mice displayed similar responses *in vitro* to alloantigen stimulation and were equally able to reject allogeneic skin transplants (123). Moreover, no deletion or anergy of autoreactive effectors was observed after combi-GAD therapy as adoptive transfer of CD25-depleted splenocytes from cured combi-GAD65$_{370–575}$-treated mice induced diabetes in NOD/SCID recipients (125). An earlier study showed that intranasal PINS administration when combined with systemic anti-CD3 successfully induced long-term reversion of diabetes in around 50% of both RIP-LCMV and NOD mice and this reversal was linked to induction of PINS-responsive Tregs (128). Indeed, treatment with *L. lactis*-based combi-PINS increases antigen-specific functional $CD4^+CD25^+Foxp3^+$ Tregs which homed to the pancreas (**Figure 2**).

Furthermore, two predictive biomarkers were discovered for therapeutic success, namely, glycemia values at onset which reflects residual beta cell mass and insulin autoantibody (IAA) positivity (124). *Post-hoc* analysis of the Diabetes Prevention Trial-Type 1 (DPT-1), where at risk children were fed high doses of insulin, also indicated that it may be necessary to select individuals with high IAA levels for antigen-specific trials with insulin (129). This consensus should probably be generalized to identifying autoantigen reactivity in patients to select appropriate antigen-specific therapies. Finally, the clinical-grade combi-PINS therapy induces functional IL-10-secreting $Foxp3^+$ ($CD25^+$ and $CD25^-$) Tregs in

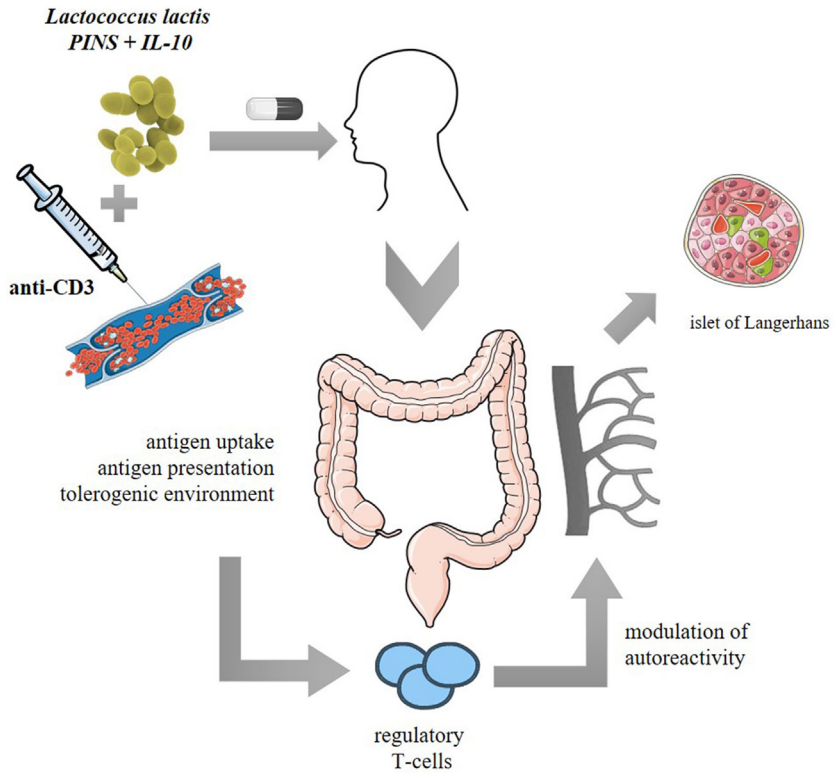

FIGURE 2 | Proposed clinical model of *Lactococcus lactis*-based vaccine or immunotherapy: oral administration of freeze-dried encapsulated biologically contained *L. lactis* modified to secrete human proinsulin (PINS) and interleukin-10 (IL-10) combined with intravenous systemic monoclonal CD3 antibodies (anti-CD3). PINS together with IL-10 may be presented to migratory CD103$^+$ dendritic cells which will home to the mesenteric lymph nodes and mediate priming of Foxp3$^+$ regulatory T cells (Tregs). The induced Tregs will proliferate and acquire tolerogenic properties when migrating back to the lamina propria and will thereafter be released in the circulation to modulate islet beta cell autoreactivity in a Foxp3- and IL-10-dependent manner.

the pancreatic draining lymph nodes and the pancreas, irrespective of metabolic outcome. On the other hand, CTLA-4$^+$ frequency among Treg subsets was only increased in mice responsive of therapy. Foxp3$^+$ Treg frequencies were increased in the periphery of treated mice compared with newly diabetic controls, especially in therapy responders, alluding to the potential of this population as an immune biomarker for therapy. Additionally, Foxp3$^+$ T cells were shown to be crucial for both induction and maintenance of *L. lactis*-based combi-PINS tolerance.

We believe the in-depth characterization of mechanisms of action of the safe *L. lactis*-based combination therapy combined with the abovementioned biomarkers for predicting therapeutic success validate this therapy as a suitable intervention for T1D that is ready for clinical testing.

TRANSLATION TO CLINIC

The preclinical efficacy data obtained from animal models are very encouraging, but it is important not to forget the significant differences with humans. Changes in dose, composition, and administration form can have substantial effects on drug potency. It is also important to keep in mind the type of animal and disease model that was used. Data from humanized mice will provide a better prediction of effects expected to be seen in human trials. On the other hand, chemically induced disease models may also not mimic the entire pathology, like DSS-induced colitis which can even happen in the absence of adaptive immunity.

Both *L. lactis*-based combination therapies, combi-PINS and combi-GAD65, hold tremendous promise as they effectively reverse T1D in an auto-antigen-specific manner without inducing general immune suppression. However, successful clinical translation requires the generation of a *L. lactis* strain suitable for patient use and the identification of certified biomarkers for both immunological and therapeutic success. Recently our group tested such a self-containing clinical-grade *L. lactis* strain, meaning chromosomal integration of human PINS and IL-10 sequences (24), in an intervention protocol with low-dose anti-CD3 in NOD mice (124). The safety profile of GM *L. lactis* strains will be of utmost importance if they are to be used to treat new-onset T1D patients, which are often young children. *L. lactis* bacteria are, as is discussed in detail above, harmless of nature and have been ingested even at high doses by healthy children, adults, elderly, as well as immune-compromised individuals and showed no health compromising issues (**Figure 1**). To date several clinical trials using live GM *L. lactis* have been completed showing clearly that treating patients with mucosal pathologies was safe and well tolerated (**Table 1**). These trials demonstrated that the *thyA*-containment system designed in 2000 was effective

TABLE 1 | Clinical studies using *Lactococcus lactis* as carriers to target mucosal delivery of heterologous proteins.

	Strain	Heterologous protein secretion	Expression system	Inflammatory condition	Administration	Clinical trial	Outcome	Reference	Clinical trial identifier
Live genetically modified *L. lactis*	*L. lactis* Thy 12	hIL-10	ThyA native promoter from *L. lactis*	Crohn's disease	Oral capsule	Phase-I trial evaluating safety and biologic containment of the transgene in patients with Crohn's disease	Treatment was safe and well tolerated, furthermore bacterial growth after passage through the GI tract was dependent on thymidine indicating the environmental containment system is effective	Preclinical data (55) Clinical data (57)	
	L. lactis AG011	hIL-10	ThyA native promoter from *L. lactis*	Ulcerative colitis	Oral capsule	Phase-IIa trial to evaluate the safety tolerability, pharmacodynamics, and efficacy of AG011 in patients with ulcerative colitis	Primary endpoints were met, confirming safety and environmental containment. However, no statistical significant effects on mucosal healing were observed	ActoGenix press release (October 9th 2009)	NCT00729872
	L. lactis AG013	hTFF1	ThyA native promoter from *L. lactis*	Oral mucositis	Oral rinse	Phase-I trial in healthy volunteers to evaluate the pharmacokinetic profile of orally delivered AG013	The PK profile showed live AG013 bacteria adhere to the oral mucosa and actively secrete protein for up to 24 h. Food intake reduced exposure while intake of a beverage lid not	ActoGcni$_x$ press release (August 22nd 2012)	
	L. lactis AG013	hTFF1	ThyA native promoter from *L. lactis*	Oral mucositis	Oral rinse	Phase-Ib trial to assess safety and tolerability of topically applied AG013 in oral mucositis in subjects receiving induction chemotherapy for the treatment of cancers of the head and neck	Treatment was safe, as no AG013 bacteria were detected in blood. Compliance was in accordance with daily dosing frequency and preliminary efficacy data were reported	Preclinical data (76) Clinical data (77)	NCT00938080
	L. lactis AG013	hTFF1	ThyA native promoter from *L. lactis*	Oral mucositis	Oral rinse	Phase-II trial to determine efficacy, safety and tolerability of AG013 in oral mucositis compared with placebo when administered three times per day	Actively recruiting, estimated primary completion date May 2020		NCT03234465
	L. lactis AG014	Anti-TNF-alpha (Certolizumab)	Not disclosed	Inflammatory bowel disease	Oral capsule	Phase-I trial studying safety and tolerability, medical endoscopic sampling methodology and characterization of the pharmacokinetic profile of oral doses of AG014 in healthy volunteers	Showed high safety and tolerance levels while also showing live AG014 were targeted to the GI tract and localized exposure of anti-TNF-alpha was efficiently measurable by endoscopic sampling	Preclinical data (67) ActoGeni$_x$ press release (October 15th 2014)	

(*Continued*)

TABLE 1 | Continued

	Strain	Heterologous protein	Linkage system	Inflammatory condition	Administration	Clinical trial	Outcome	Reference	Clinical trial identifier
Non-live non-genetically modified *L. lactis*	Bacterium-like particles derived from inactivated *L. lactis*	RSV fusion protein F	Protein domain fused to heterologous protein will bind non-covalently to lactococcal peptidoglycan	Respiratory syncytial virus	Intranasal spray	Phase-I trial to assess the safety. reactogenicity and tolerability of two intranasal dose levels of SynGEM® in healthy volunteers	Estimated primary completion date December 2017	Preclinical data (131) Mucosis press release (November 7th 2016)	NCT02958540

GI, gastrointestinal; hIL-10, human interleukin-10; hTFF1, human trefoil factor 1; L. lactis, Lactococcus lactis; PK, pharmacokinetic; RSV, respiratory syncytial virus; TNF, tumor necrosis factor.

at restricting environmental dissemination and pharmacokinetic assessment confirmed an adequate formulation for human use was found. The pharmacokinetic profile of *LL-IL10* was also tested in healthy and colitic mice and even in cases of severe intestinal inflammation (and therefore gut leakiness) no *L. lactis* were found in the circulation (130). Toxicity studies performed in mice and primates showed no evidence for anti-hIL10 antibodies and the no observed adverse effects level (NOAEL) was the highest dose given (130). Of note, *L. lactis* are also being tested in a phase-I trial (NCT02958540) as non-live non-GM carriers to deliver antigens to the nasal mucosa as a vaccination strategy against respiratory syncytial virus (131) (**Table 1**).

Clinical trial feasibility will rely on bringing preclinically tested laboratory strain of *L. lactis* from the bench to bedside. One hurdle to overcome is stable storage of large amounts of temperature-sensitive *L. lactis*. Freeze-drying, or lyophilization, reduces water activity in bacteria and allows long-term and low-cost storage at temperatures above freezing (132). This formulation also improves passage through the GIT; however, it can also significantly reduce viability. On the other hand, a different modification, namely, the accumulation of intracellular trehalose to improve bile resistance, concurrently improved viability under this formulation (20). Such technical adaptations are the cornerstone of successful therapeutic clinical translation.

The obtained safety data are reassuring in moving forward with clinical testing of live GM *L. lactis* bacteria. Furthermore, significant progress has already been made to develop optimal formulations suitable for human use.

CONCLUSION

Lactococcus lactis have evolved from agents used in the food industry into qualified vehicles for mucosal drug delivery. However, several technological advancements were necessary for this transition, such as the identification of a strong constitutive promoter as well as several inducible expression systems. Furthermore, heterologous protein expression was shown to be possible at a handful of cellular locations. These modifications put the *L. lactis* on the map as an extremely versatile vehicle. Nonetheless, the industrial convenience is only transcended by the safety profile these bacteria have demonstrated in clinical trials. A proven effective environmental containment system, through replacement of the lactococcal *thyA* gene, is reassuring with respect to concerns regarding clinical use of GM organisms. Therefore, it is possible to carefully move forward with the substantial successful preclinical results obtained using GM *L. lactis* as immunotherapeutic tools.

AUTHOR CONTRIBUTIONS

DC and CG drafted the manuscript and CM critically revised the manuscript.

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Dexamethasone-Induced Myeloid-Derived Suppressor Cells Prolong Allo Cardiac Graft Survival through iNOS- and Glucocorticoid Receptor-Dependent Mechanism

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How to induce immune tolerance without long-term need for immunosuppressive drugs has always been a central problem in solid organ transplantation. Modulating immunoregulatory cells represents a potential target to resolve this problem. Myeloid-derived suppressor cells (MDSCs) are novel key immunoregulatory cells in the context of tumor development or transplantation, and can be generated *in vitro*. However, none of current systems for *in vitro* differentiation of MDSCs have successfully achieved long-term immune tolerance. Herein, we combined dexamethasone (Dex), which is a classic immune regulatory drug in the clinic, with common MDSCs inducing cytokine granulocyte macrophage colony stimulating factor (GM-CSF) to generate MDSCs *in vitro*. Addition of Dex into GM-CSF system specifically increased the number of $CD11b^{+}$ $Gr\text{-}1^{int/low}$ MDSCs with an enhanced immunosuppressive function *in vitro*. Adoptive transfer of these MDSCs significantly prolonged heart allograft survival and also favored the expansion of regulatory T cells *in vivo*. Mechanistic studies showed that inducible nitric oxide sythase (iNOS) signaling was required for MDSCs in the control of T-cell response and glucocorticoid receptor (GR) signaling played a critical role in the recruitment of transferred MDSCs into allograft through upregulating CXCR2 expression on MDSCs. Blockade of GR signaling with its specific inhibitor or genetic deletion of iNOS reversed the protective effect of Dex-induced MDSCs on allograft rejection. Together, our results indicated that co-application of Dex and GM-CSF may be a new and important strategy for the induction of potent MDSCs to achieve immune tolerance in organ transplantation.

Keywords: myeloid-derived suppressor cells, immune tolerance, dexamethasone, iNOS, glucocorticoid receptor

INTRODUCTION

The ultimate goal for organ transplantation is to achieve long-term acceptance without the need for immunosuppressive drugs (1). Cellular immunotherapy is a promising alternative for preventing allograft rejection and adoptive transfer of immune cells has been tried to establish immune tolerance in animal models (2–4). Our previous results showed that adoptive transfer of adaptive immune cells, regulatory T cells (Treg), can help to establish mixed bone marrow chimeras and lead

to the prolongation of intestinal allograft survival (5). However, the possibility of adoptive transfer of other immune cells such as innate immune cells to achieve immune tolerance has not been fully elucidated.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of innate immune cells generated from immature myeloid cells in the bone marrow and were first characterized in tumor-bearing mice or in patients with cancer (6). With the increased understanding of MDSC development, it is now clear that pathological situations such as inflammation and tumor development can lead to a partial block in the differentiation of immature myeloid cells into mature myeloid cells, resulting in the expansion of MDSCs (6). In mice, the phenotype of MDSCs was CD11b (also known as integrin-αM) and granulocyte differentiation antigen-1 (Gr-1), and in humans, their phenotype is $Lin^{-}HLA^{-}DR^{-}CD33^{+}$ or $CD11b^{+}CD14^{-}CD33^{+}$ (6). Once generated in a pathological context, MDSCs were able to control T-cell immune response through the upregulated expression of immune suppressive factors such as arginase, inducible nitric oxide synthase (iNOS), nitric oxide, and reactive oxygen species. Thus, despite early evidence of MDSCs in the control of tumor development, accumulating results have also suggested a critical role of MDSCs in the amelioration of allogenic transplantation based on their immune suppressive function (4, 7). Manipulating both the development and function of MDSCs has been suggested to be a potential therapeutic target to achieve immune tolerance (8). However, although numerous studies have identified multiple signaling pathways to contribute to the induction and function of MDSCs *in vivo* and *in vitro*, none of them have been applied to clinical trials possibly due to the instability of MDSCs *in vivo*.

Myeloid-derived suppressor cells can be induced from bone marrow progenitors *in vitro*, and the most common way to induce MDSCs *in vitro* is the use of granulocyte macrophage colony stimulating factor (GM-CSF) at high concentrations for 4 days (9). In addition, *in vitro*-generated MDSCs by GM-CSF have also been shown to prolong the survival of pancreatic islet allograft upon adoptive transfer (7), which provides promising prospects of cellular immunotherapy based on *in vitro*-generated MDSCs in the context of transplantation. Dexamethasone (Dex) belongs to the family of glucocorticoid and has been widely used in treating inflammatory disorders (10). Dex can not only inhibit the production of inflammatory cytokines *in vivo* but also modulate the phenotype and/or function of both classic innate and adaptive immune cells such as dendritic cells (11), macrophages (12), and Treg (13). However, the role of Dex in the modulation of MDSCs has not been fully addressed. In the present study, we sought to determine whether the use of Dex together with GM-CSF can promote the differentiation of MDSCs *in vitro* with an enhanced immunosuppressive ability, and also explore whether adoptive transfer of these *in vitro*-induced MDSC can help to achieve immune tolerance *in vivo*.

MATERIALS AND METHODS

Mice

BALB/c, C57BL/6 (B6), and $CD45.1^{+}$ B6 mice were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). All the mice were bred and maintained in the condition of specific pathogen-free. Male 8- to 10-week-old littermate mice were used for experiments. iNOS knockout (KO) mice (B6 background, CD45.2) were purchased from Jackson Lab. All the animal experiments were carried out following the Guidelines for the Care and Use of Laboratory Animals of Nanjing University and were approved by the ethical review committee of Nanjing University.

Cell Culture

Bone marrow cells were acquired from tibias and femurs of B6 mice (CD45.1 or CD45.2) as described previously (14). Tibias and femurs were removed using sterile techniques, and bone marrow was flushed with PBS. Red blood cells were lysed with ammonium chloride. Total bone marrow cells were planted into 100-mm dishes (Corning, USA) for 2 h. The non-adherent cells were collected and were further cultured in the presence of 50 ng/ml GM-CSF, with or without Dex at different concentration as indicated in 60-mm-diameter dish (Corning, USA) in 4 ml of complete RPMI 1640 medium (containing 10% heat-inactivated FBS, 2mM L-glutamine, 10 mM HEPES, 20 μM 2-ME, 150 U/ml streptomycin and 200 U/ml penicillin) at 37°C, 5% CO_2 for 4 days.

T Cell Proliferation Assay

Splenic cells were labeled with 2 μM CFSE for 5 min in PBS at 37°C and were then washed with RPMI 1640. The labeled cells were further cultured with ConA (2 μg/ml) (15) in the presence of different doses of MDSCs as indicated for 72 h. Cell proliferation was assessed by flow cytometry after stained with anti-CD4 or anti-CD8 mAb.

Flow Cytometry Antibodies and Reagents

Anti-mCD11b-FITC, anti-mCD11b-PE, anti-mGr-1-PE-Cy5, anti-mCD31-PE, anti-mF4/80-PE, anti-mCD11c-PE, anti-mI-Ab-PE, anti-mCD115-PE, anti-mCD274-PE, anti-mCD80-PE, anti-mCD86-FITC, anti-mCD115-PE, anti-mCD62L-PE, anti-mIgG-PE, anti-mIgG-FITC, and anti-mFoxp3-PE were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Anti-mCD8-PE-Cy5, anti-mCD4-FITC, and anti-mCD4-PE were purchased from eBioscience (San Diego, CA, USA). Anti-mCD45.2-PE-Cy5, anti-mCD45.1-APC, anti-mLy6G-PE, and anti-mCD11b-FITC were purchased from biolegend. Recombinant mouse GM-CSF was purchased from PeproTech (Rock Hill, NJ, USA). Dex, RU486 (16), bacterial lipopolysaccharide (LPS; *E. coli* 055:B5), and NG-monomethyl-L-arginine (L-NMMA) (17) were purchased from Sigma-Aldrich. Anti-CXCR2 mAb (16) and IgG isotype were purchased from R&D Systems (Minneapolis, MN, USA).

NO Production Assay

The Griess reaction was used to measure nitrite production. Equal volumes of culture supernatants (100 μl) were incubated in 96-well plate with Griess reagent at room temperature for 10 min, and absorbance was determined at 550 nm in a microplate reader (Bio-Rad). Nitrite concentrations were calculated by comparing the absorbance values for the test samples to a standard curve generated by a serial dilution of sodium nitrite.

Cardiac Allograft Transplantation

Hearts from Balb/c donors were transplanted heterotopically into the abdominal cavity of B6 recipients. Anastomoses were performed between the donor ascending aorta/pulmonary arties and the recipient descending aorta/inferior vena cava. The function of the cardiac graft was monitored daily by palpation through the abdominal wall. Graft loss was defined as no palpable beat. In some experiments, grafts were excised, fixed in 10% buffered formaldehyde, paraffin embedded, sectioned (3 μm), and stained with hematoxylin and eosin (H&E) as we described previously (5).

Cell Isolation, Flow Cytometry, and Adoptive Transfer

Splenic cells and peripheral white blood cells were prepared as we described previously (5). For isolation of infiltrated cells in cardiac allografts, allografts were cut into small pieces and then digested for 40 min at 37°C with 400 U/ml collagenase IV (Sigma-Aldrich), 10 mM HEPES, and 0.01% DNase I (MP Biomedicals) in HBSS. Digested suspensions were passed through a nylon mesh. Then cells were collected after centrifugation and re-suspended in FACS staining buffer for cell surface marker staining. Samples were analyzed on a Beckman Coulter Epics XL benchtop FCM (Beckman Coulter) with FCS express software (*De Novo* Software, Thornhill, ON, Canada). $CD11b^+$ $Gr\text{-}1^{int/low}$ cells were sorted after *in vitro* culture using a FACSAria II (BD Biosciences) and were adoptively transferred into B6 recipient immediately after cardiac allograft transplantation (3×10^6 per mouse, i.v.).

Quantitative Real-time PCR

Total RNA of the indicated cells was extracted using RNAeasy mini kit (Qiagen) and reverse transcribed with Superscript II (Qiagen) according to the manufacturer's instructions. The cDNA served as template for the amplification of target genes and the housekeeping gene (*HPRT*) by real-time PCR. Target gene expression was calculated using the comparative method for relative quantification upon normalization to *HPRT* gene expression. Primer sequences:

HPRT Forward: AGTACAGCCCCAAAATGGTTAAG,
Reverse: CTTAGGCTTTGTATTTGGCTTTTC;
iNOS Forward: CACCAAGCTGAACTTGAGCG,
Reverse: CGTGGCTTTGGGCTCCTC;
GRα Forward: ACTGCCCCAAGTGAAAACAGA,
Reverse: GCCCAGTTTCTCCTGCTTAATTAC;
CXCR2 Forward: TCACAAACAGCGTCGTAGA,
Reverse: GACAGCATCTGGCAGAATAG.

Statistical Analysis

Data were reported as Mean ± SEM. Student's *t*-test was used to compare the differences between two groups, and one-way or two-way ANOVA analysis was used for comparison among multiple groups. Survival studies were analyzed with the log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Dex Enhances the Differentiation and Suppressive Capacity of MDSCs Induced by GM-CSF *In Vitro*

To address the role of Dex in the induction of MDSCs, MDSCs were generated from bone marrow cells in the presence of GM-CSF as previously described, with or without different concentrations of Dex for 4 days. As shown in **Figure 1A**, GM-CSF or GM-CSF with different concentrations of Dex all induced $CD11b^+$ $Gr\text{-}1^+$ cells. Interestingly, we found Dex could increase the percentage and cell number of $CD11b^+Gr\text{-}1^{int/low}$ cells but not $CD11b^+Gr\text{-}1^{hi}$ cells in a dose-dependent manner (**Figures 1A,B**). Based on the increased percentage and cell number of $CD11b^+Gr\text{-}1^{int/low}$ cells induced by GM-CSF and Dex, $CD11b^+Gr\text{-}1^{int/low}$ cells were gated to further analyze cell surface markers. The expression of CD115, F4/80, and CD11c were identical between the cells treated with GM-CSF or GM-CSF + Dex (**Figure 1C**), indicating Dex treatment does not alter cell phenotype. Other surface markers indicative for MDSCs suppressive action, including CD80 and CD86 were significantly decreased in cells induced by Dex. CD62L, which enables MDSCs homing into lymphoid organs, was slightly increased on the surface of MDSCs induced by Dex and GM-CSF (**Figure 1C**).

To directly assess the suppressive function of MDSCs induced by GM-CSF or GM-CSF + Dex, cell proliferation of T cells stimulated by ConA was determined in the presence of the induced MDCs at different ratios. GM-CSF-induced MDSCs can inhibit the proliferation of $CD8^+$ and $CD4^+$ T cells in a dose-dependent manner, while GM-CSF + Dex-induced MDSCs displayed stronger suppressive capacity (**Figure 1D**). MDSCs induced by GM-CSF + Dex can inhibit T cell proliferation even in lower ratios (**Figures 1E,F**). Therefore, these results suggested Dex can enhance the immunosuppressive function of GM-CSF-induced MDSCs *in vitro*.

The Enhanced Immunosuppressive Function of MDSCs Induced by Dex Is Mediated by iNOS *In Vitro*

To further explore the mechanisms accounting for the enhanced immunosuppressive function seen in GM-CSF + Dex-induced MDSCs, immunosuppressive molecules were assessed in MDSCs induced by GM-CSF or GM-CSF + Dex in the presence or absence of splenocytes. There were no significant differences in potential relevant genes, including Arg1, Nox2, COX2, HO-1, TGF-β, and IDO between MDSCs induced by GM-CSF and by GM-CSF + Dex (data not shown), while the expression of iNOS in GM-CSF + Dex-induced MDSCs was significantly higher than that in GM-CSF-induced MDSCs (**Figure 2A**). Next, we assessed the level of NO production in MDSCs co-cultured with T cells activated by ConA. The NO production was also significantly higher in the culture system with MDSCs induced by GM-CSF + Dex (**Figure 2B**), suggesting the critical role of iNOS pathway in the immunosuppressive function of Dex-induced MDSCs. Moreover, addition of a nitric oxide synthase inhibitor, L-NMMA, which can specifically block NO production, significantly reversed the inhibitory effects of MDSCs induced by GM-CSF or GM-CSF + Dex on T cell proliferation

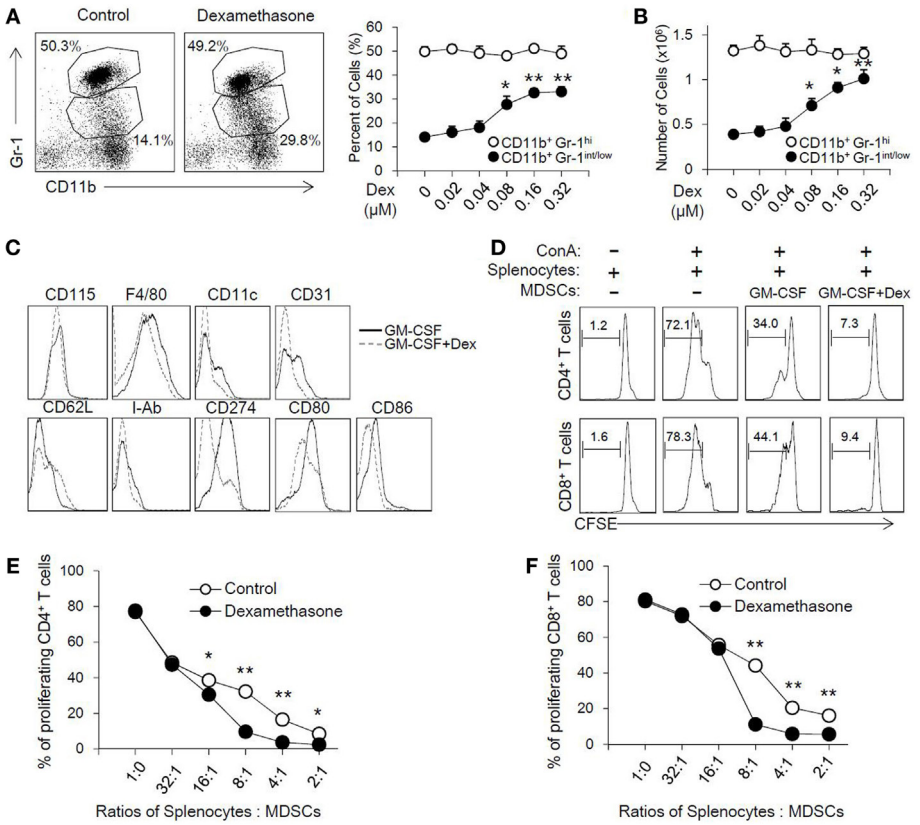

FIGURE 1 | Dexamethasone (Dex) increases the cell number of CD11b$^+$ Gr-1$^{int/low}$ Myeloid-derived suppressor cells (MDSCs) with an enhanced suppressive function *in vitro*. MDSCs were induced from bone marrow cells in the presence of granulocyte macrophage colony stimulating factor (GM-CSF), with or without Dex for 4 days. The percentage **(A)** and cell number **(B)** of MDSCs induced by GM-CSF or GM-CSF + Dex were shown. The phenotype markers **(C)** of MDSCs induced *in vitro* were also summarized by flow cytometry. **(D)** CFSE-labeled splenic cells (2×10^5 per well) were stimulated by ConA (2 μg/ml), and MDSCs induced by GM-CSF or GM-CSF + Dex were added at different ratios between splenic cells and MDSCs for 72 h. The pattern of proliferation was examined by flow cytometric analysis of CFSE dilution in the gated CD4$^+$ and CD8$^+$ T cells. The percentages indicated CFSE dilution in the gated T cell population (number of splenic cells and MDSCs: 2×10^5 and 0.25×10^5). The inhibitory rates in CD4$^+$ T cells **(E)** and CD8$^+$ T cells **(F)** were calculated based on the proliferation rates determined by flow cytometry analysis. The data (Mean ± SEM) are representative from three independent experiments (*$P < 0.05$, **$P < 0.01$).

in a dose-dependent manner (**Figures 2C,D**). In addition, there was no significant difference in the immunosuppressive function between iNOS-deficient MDSCs induced by GM-CSF or GM-CSF + Dex, both of which displayed significantly decreased inhibitory function (**Figures 2E,F**). Inhibition of IDO, Cox2, and Arg1 with their corresponding inhibitors also failed to recover T cell proliferation in the culture system with MDSCs induced by GM-CSF alone or GM-CSF + Dex (data not shown). Taken together, all these results clearly showed that iNOS pathway is responsible for the enhanced immunosuppressive function of MDSCs induced by GM-CSF + Dex in vitro.

Adoptive Transfer of MDSC-Dex Prolongs Cardiac Allograft Survival in an iNOS-Dependent Manner

To examine the role of MDSCs in allograft rejection *in vivo*, GM-CSF-induced MDSCs (MDSC-control) or GM-CSF + Dex-induced MDSCs (MDSC-Dex) were transferred to allogeneic cardiac transplant mice. Adoptive transfer of MDSC-Dex significantly prolonged cardiac allograft survival when compared to that of MDSC-control (**Figures 3A,B**). Previous results showed iNOS pathway accounted for the enhanced immunosuppressive function of MDSC-Dex *in vitro*. To explore whether iNOS pathway is also required for MDSC-Dex mediated suppressive function *in vivo*, MDSC isolated from iNOS KO mice were induced by GM-CSF + Dex *in vitro* and were adoptively transferred to allogeneic cardiac transplant mice. Absent of iNOS pathway in MDSC induced by GM-CSF + Dex significantly reversed the effect of MDSC-Dex on allograft survival (**Figure 3C**). The infiltration of immune cells including CD8$^+$ T, CD4$^+$ T, and macrophages were also significantly increased in iNOS KO mice transferred with MDSCs induced by GM-CSF + Dex (**Figure 3D**). Therefore, NO is required for Dex-induced MDSC amelioration of allograft immune response both *in vivo* and *in vitro*.

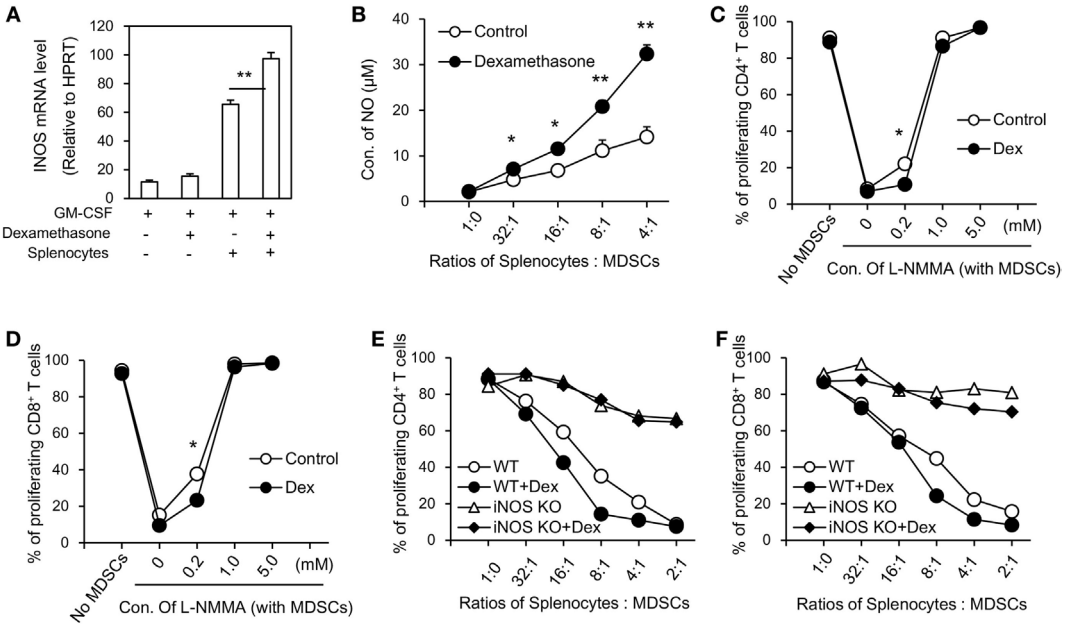

FIGURE 2 | Dexamethasone (Dex) enhances the inhibitory function of myeloid-derived suppressor cells (MDSCs) through iNOS pathway. **(A)** MDSCs (C57BL/6, CD45.1) induced by granulocyte macrophage colony stimulating factor (GM-CSF) or GM-CSF + Dex were cocultured with splenic cells (C57BL/6, CD45.2) in the presence of conA (2 μg/ml) at a ratio of 1:1 for 3 days, and MDSCs were harvested by flow cytometry based on the surface staining of CD45.1. The mRNA expression of *iNOS* in harvested MDSCs was determined by RT-PCR. **(B)** MDSCs induced *in vitro* were cocultured with CFSE-labeled splenic cells (2×10^5 per well) stimulated by ConA (2 μg/ml) at different ratios for 72 h and the NO levels in the coculture medium were assessed. iNOS inhibitor L-NMMA at different concentrations were added into T cells and MDSCs coculture system for 72 h. Percentage of proliferating $CD4^+$ T cells **(C)** and $CD8^+$ T cells **(D)** were shown. MDSCs induced from iNOS knockout (KO) mice by GM-CSF or GM-CSF + Dex were added to CFSE-labeled splenic cell proliferation assay. iNOS deficiency significantly reversed the inhibitory effect of MDSCs induced by GM-CSF + Dex on $CD4^+$ T cells **(E)** and $CD8^+$ T cells **(F)**. Data are shown as Mean ± SEM. All the data are composite of three independent experiments (*$P < 0.05$, **$P < 0.01$).

MDSCs Induced by Dex Displayed an Enhanced Migration into Allograft through Upregulating the Expression of Glucocorticoid Receptor (GR) and CXCR2

The migration of MDSCs into allograft is critical for its immunosuppressive function, as well as for the prolongation of allograft survival (18). To explore whether the prolongation of allograft seen in Dex-MDSCs treated mice was due to an enhanced migration of MDSCs, we first assessed the expression of CXCR2, which was implicated in mediating MDSCs recruitment into local inflammatory and tumor sites (19, 20). We found the expression of CXCR2 was significantly upregulated in MDSCs induced by GM-CSF + Dex (**Figure 4A**), and MDSCs induced by GM-CSF + Dex also displayed a significantly increased infiltration into allograft (**Figure 4B**). In addition, anti-CXCR2 treatment significantly reduced the number of MDSCs in allograft, resulting in a decreased allograft survival in both MDSCs-Dex group and MDSCs-control group (**Figures 4B,C**). Thus, Dex promotes the migration of MDSCs into allograft through upregulating the expression of CXCR2.

Next, we explored how Dex modulates the expression of CXCR2 on MDSCs. GR signaling is regarded to be essential for GC effects, and we found Dex treatment significantly upregulated the expression of GR in MDSCs both at the mRNA and protein levels (**Figures 4D,E**). Moreover, the use of a GR specific inhibitor, RU486, decreased the expression of CXCR2 on MDSCs (**Figure 4F**). RU486 treatment also reduced the infiltration of MDSCs in allograft (**Figure 4G**), leading to a diminished allograft survival (**Figure 4H**). Taken together, these results suggest Dex promotes the migration of MDSCs into allograft through GR-dependent pathway.

Dex-Induced MDSCs Promote $CD4^+$ $Foxp3^+$ Treg Expansion

Previously, MDSCs have been shown to mediate $CD4^+$ $Foxp3^+$ Treg development in mouse tumor models *in vivo* (21, 22). Recent studies further suggested that MDSCs can induce Treg development in animal transplantation model (23) and renal transplant patients (24). To address whether MDSCs in our model can also promote the expansion of Treg *in vivo*, the percentages of Treg in the allografts, the peripheral blood and the spleens of mice receiving MDSC-control or MDSC-Dex were analyzed at post transplantation day 7. As shown in **Figure 5A**, the percentage of Treg in the allografts of mice receiving MDSC-Dex was significantly higher than that receiving MDSC-control. Similar results were also found in the peripheral blood and the spleen (**Figures 5B,C**). To direct assess the effect of MDSCs on the expansion of Treg, we cocultured MDSCs induced by GM-CSF or GM-CSF + Dex with

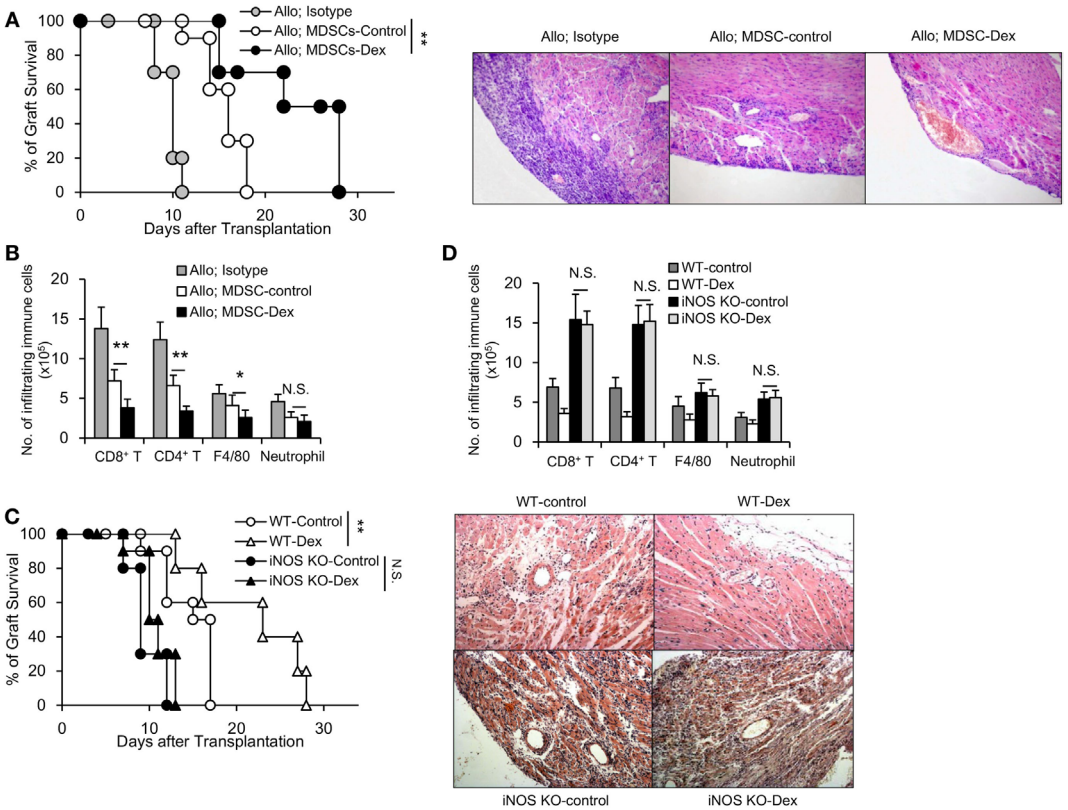

FIGURE 3 | Adoptive transfer of granulocyte macrophage colony stimulating factor (GM-CSF) + dexamethasone (Dex)-induced Myeloid-derived suppressor cells (MDSCs) prolongs cardiac allograft survival in an iNOS-dependent manner. **(A)** Cardiac allograft survival (BALB/c donor hearts to C57BL/6J recipient mice) from mice transferred with sorted CD11b^{+}Gr-1$^{int/low}$ MDSCs (3×10^{6} per mouse, i.v.) induced by GM-CSF or GM-CSF + Dex immediately after cardiac allograft transplantation was monitored ($n = 8$ per group), and H&E staining of representative sections at postoperative day 7 from mice transferred with MDSCs were shown. **(B)** Numbers of infiltrating immune cells in cardiac allografts were assessed by flow cytometry. **(C)** Deficiency of iNOS reversed the protective function of Dex-induced MDSCs on cardiac allograft in recipient mice ($n = 8$ per group). **(D)** The infiltration of immune cells in cardiac allografts were analyzed in WT or iNOS knockout (KO) mice transferred with MDSCs induced by GM-CSF or GM-CSF + Dex. $^{*}P < 0.05$, $^{**}P < 0.01$, N.S., not significant.

purified CD4^{+} T cells from the spleen for 3 days. As shown in **Figures 5D,E**, MDSCs induced by Dex + GM-CSF significantly enhanced the pool of Treg. Taken together, all these results suggested MDSCs induced by GM-CSF + Dex favors the expansion of Treg.

DISCUSSION

Historically, developing therapeutic strategies of transplantation tolerance mainly focuses on targeting adaptive immune response, based on the findings that T cells are both necessary and sufficient to induce allograft rejection (25–28). Deletion of activated T cells and development of Treg are the two main mechanisms. However, deletion of activated T cells is necessary but not sufficient for the induction of immune tolerance and Treg also do not seem to contribute to immune tolerance during the early phase (29, 30). Modulation of innate immune cells may be a candidate to resolve this problem. Previous studies identified that classic antigen-presenting cell dendritic cells can serve as a potent innate immune cell population which promotes transplantation tolerance through inducing antigen-specific Treg (31–35). Recently, accumulating evidence from animal models also suggest a great potential of MDSCs for preventing allograft rejection (6, 36, 37), which may be a new potential therapy target on innate immune cells to control graft rejection in the clinic.

To develop a clinically acceptable method through manipulating MDSCs, *in vitro* induction of potent and stable MDSCs has been tried (6, 7). Dex is a classic drug used in clinical trials to ameliorate immune-mediated allograft rejection associated with inflammation, and can modulate immune cell phenotype and function (11–13). Recently, systemic administration of Dex has been shown to modulate MDSCs expansion *in vivo* both in skin transplantation (16) and renal disease model (38). Dex can also favor the migration of MDSCs into inflammatory sites to control immune response (16). In addition, Dex modulates MDSCs immunosuppressive function through GR signaling in an immunological hepatic injury model (39). Therefore, Dex is an important regulator of MDSCs expansion and/or function. However, since Dex can also modulate GR signaling in other cells (40, 41), systemic injection of Dex may not be an ideal way to manipulate MDSCs in future clinical trials. In our study, we used Dex combined with classic MDSC induction cytokine GM-CSF

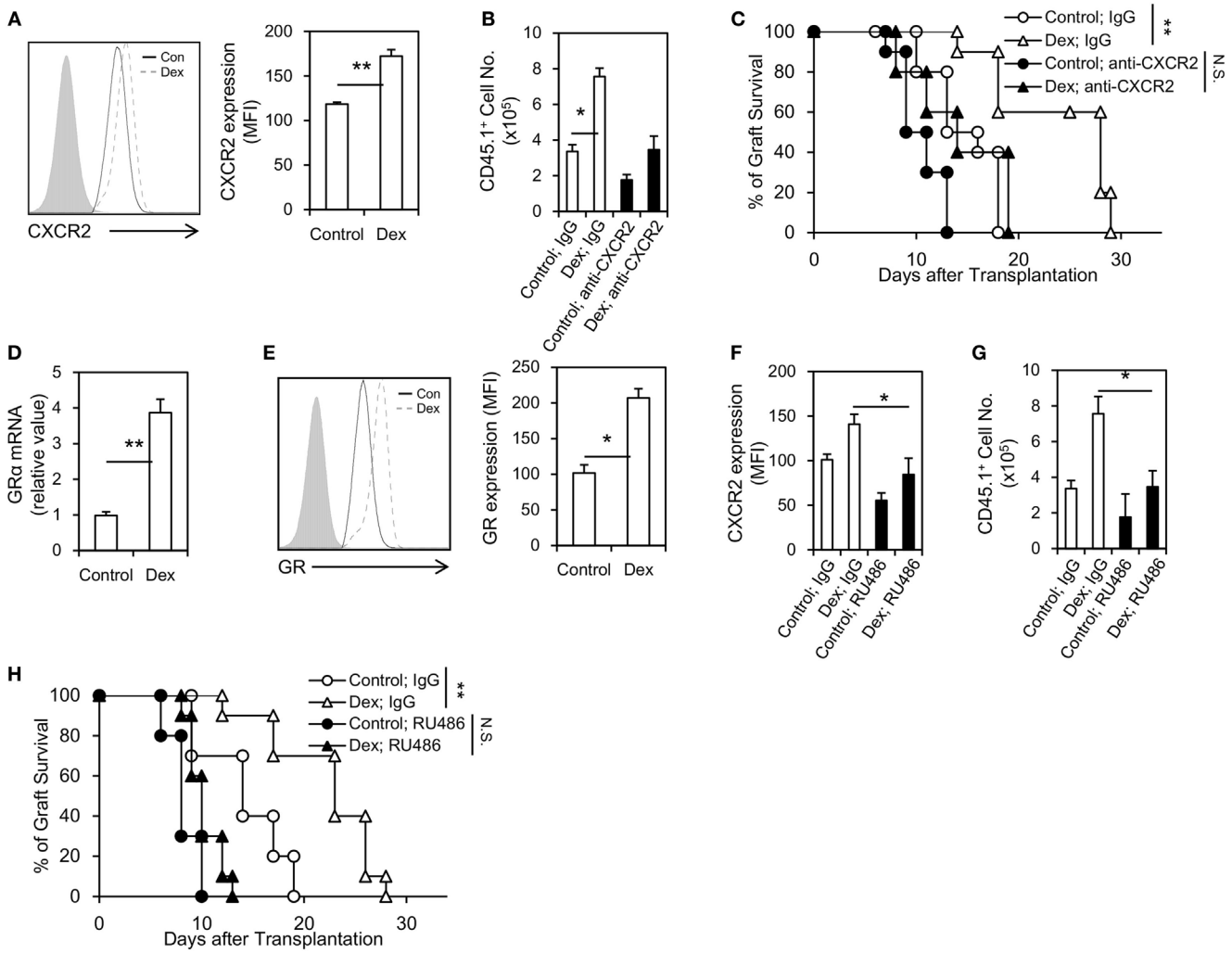

FIGURE 4 | Dexamethasone (Dex) treatment promotes Myeloid-derived suppressor cells (MDSCs) migration into cardiac allograft through upregulating the expression of glucocorticoid receptor (GR) and CXCR2. CXCR2 expression **(A)** in MDSCs induced by granulocyte macrophage colony stimulating factor (GM-CSF) or GM-CSF + Dex was determined by flow cytometry, and the mean fluorescent intensity (MFI) of CXCR2 was summarized. Allograft recipient mice (CD45.2, C57BL6/J) were administered 50 μg neutralizing anti-CXCR2 mAb or IgG isotype control *via* i.v. injection 1 h before transplantation, and then MDSCs induced by GM-CSF or GM-CSF + Dex (3×10^6 per mice, i.v.) from CD45.1 mice (C57BL6/J) were adoptively transferred into these recipients. The infiltrating donor $CD45.1^+$ $CD11b^+$ $Gr\text{-}1^+$ cell number **(B)** and graft survival **(C)** were determined ($n = 8$). Dex treatment upregulated the expression of GR in MDSCs both at the protein **(D)** and mRNA levels **(E)**. The GR antagonist RU486 efficiently diminished the CXCR2 expression on MDSCs **(F)** and decreased the infiltration of donor $CD45.1^+$ $CD11b^+$ $Gr\text{-}1^+$ MDSCs in cardiac allograft **(G)**. RU486 treatment (10 mg/kg, i.p., 1 h before transplantation) significantly diminished graft survival in recipient received MDSCs induced by GM-CSF + Dex **(H)**. Data (Mean ± SEM) are representative of two independent experiments ($n = 5$–10 in each group). $^*P < 0.05$, $^{**}P < 0.01$, N.S., not significant.

to generate the differentiation of MDSCs *in vitro*. We found Dex can specifically increase the cell number of $CD11b^+$ $Gr\text{-}1^{int/low}$ MDSCs *in vitro* with more powerful immune suppressive function on T cell proliferation. Mechanistic study showed that iNOS pathway is required for the immune regulatory function of Dex-induced MDSCs as significantly increased production of NO was found in Dex-induced MDSCs system. In addition, inhibition of NO production with genetic deletion of iNOS or chemical inhibitor blocked the immunosuppressive function of Dex-induced MDSCs *in vitro*. Consistent with these *in vitro* results, adoptive transfer of Dex-induced MDSCs significantly prolongs the survival of cardiac allograft *in vivo*.

Glucocorticoid receptor is one of the key mechanisms for the effect of Dex on MDSCs. Modulation of GR by Dex can suppress HIF1α and HIF1α-dependent glycolysis in MDSCs which promotes the immune suppressive activity of MDSCs (39). On the other hand, pharmacological inhibition of GR by RU486 can also suppress the function of MDSCs, leading to the diminished allo skin graft survival (16). To explore whether GR signaling is also involved in our study, we first assessed the GR level in MDSCs. We found GR expression was significantly increased in Dex-induced MDSCs, indicating the importance of GR signaling in Dex-induced MDSCs. The migration of MDSCs into inflammation site, i.e., the allograft in transplantation model, mainly

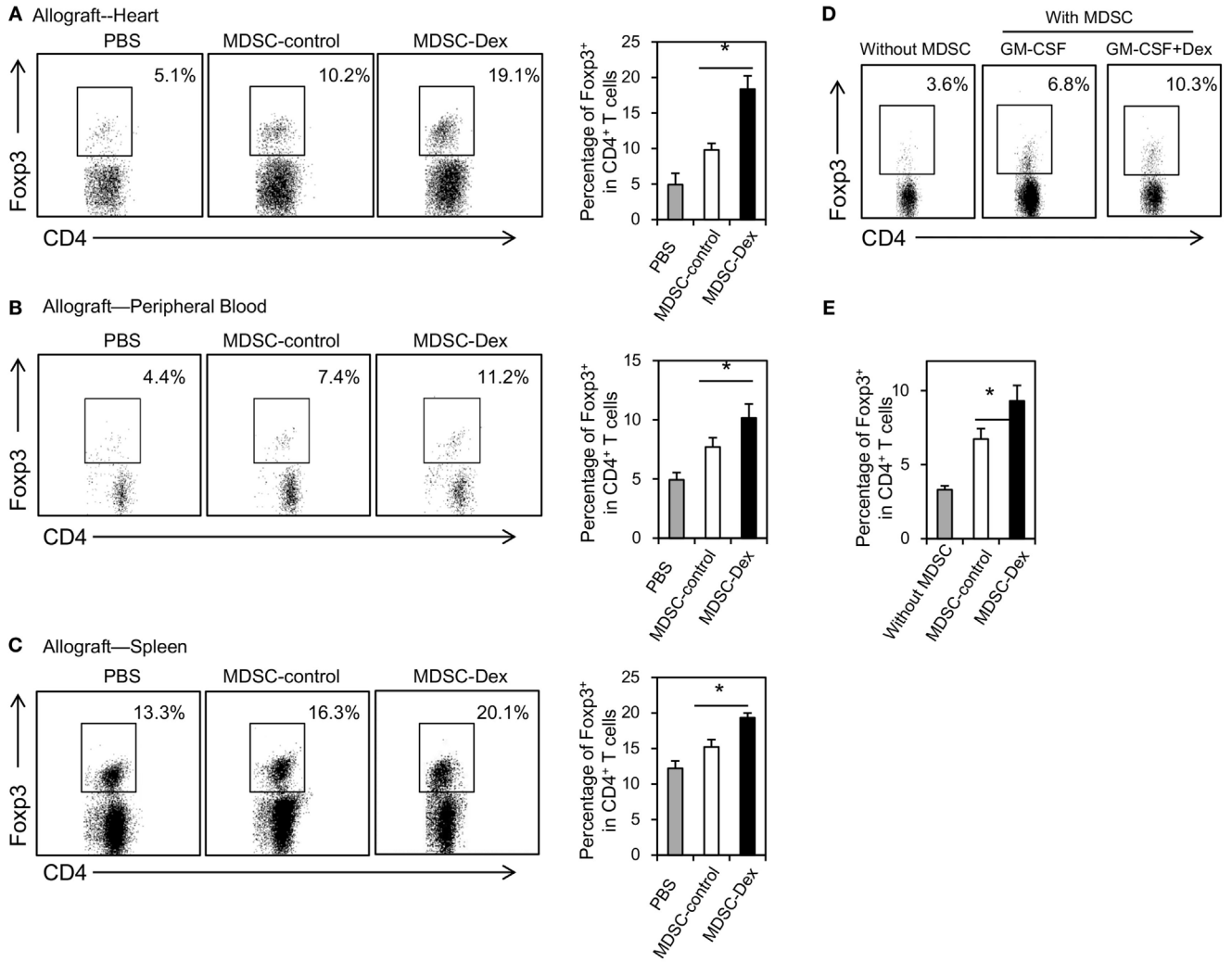

FIGURE 5 | Adoptive transfer of myeloid-derived suppressor cells (MDSCs) induced by granulocyte macrophage colony stimulating factor (GM-CSF) and dexamethasone (Dex) favors the expansion of CD4+ Foxp3+ regulatory T cells (Treg) *in vivo*. MDSCs induced by GM-CSF or GM-CSF + Dex (3×10^6 per mice) were adoptively transferred into recipient mice *via* the tail vein immediately after cardiac allograft transplantation. On post transplantation day 7, recipient mice were euthanized and the percentages of CD4+ Foxp3+ Treg in allograft **(A)**, peripheral blood **(B)**, and spleen **(C)** were assessed. **(D,E)** MDSCs (CD45.2, C57BL6/J) induced by GM-CSF or GM-CSF + Dex mice were cocultured with CD4+ T cells (CD45.2, C57BL6/J) purified by flow cytometry from the spleen at a ratio of 1:4 in the presence of anti-CD3 (5 μg/ml)/CD28(2 μg/ml) for 3 days. The percentages of Foxp3+ cells in CD4+ T cells were assessed. Data are Mean ± SEM. All the data are composite of three independent experiments (n = 3–5 in each group). P values were determined by Student's t-test. *$P < 0.05$, N.S., not significant.

depends on surface expression of CXCR2, which also plays a critical for immune tolerance. We found Dex treatment increased the expression of CXCR2 on MDSCs. Importantly, blockade of CXCR2 with a CXCR2 mAb or blockade of GR with its specific inhibitor RU486 significantly decreased the expression of CXCR2 on MDSCs and also recruitment of MDSCs into allograft, leading to a diminished survival of allograft survival. Taken together, these results demonstrated that GR signaling is required for the immune regulation of Dex-induced MDSCs.

Myeloid-derived suppressor cells and Treg are both important immune regulator cells in the context of organ transplantation (42). Since Treg or MDSCs alone was not sufficient to induce long-term tolerance, it was attractive to investigate interaction between MDSCs and Treg. Indeed, several studies have shown MDSCs promote the development and homeostasis of Treg in tumor (21, 22, 43) and organ transplantation (1, 18). Moreover, the interaction of Dex, MDSCs, and Treg has been identified in a focal segmental glomerulosclerosis mice mode (38). Both systemic administration of Dex or adoptive transfer of MDSCs can increase the percentages of Treg in the spleen and the kidney, and pharmacological depletion of MDSCs resulted in decreased levels of Treg (38). However, this study did not assess the direct effect of MDSCs on Treg expansion *in vitro*, and the renal injury itself can trigger strong expansion of Treg expansion (38). Thus, it is possible that depletion of MDSCs may lead to the changes of other cells which have an indirect impact on Treg expansion. We also investigated whether adoptive transfer of Dex-induced MDSCs can have an impact on Tregs *in vivo*. Consistent with

other studies, adoptive transfer of Dex-induced MDSCs can favor the expansion of Treg both systemically and in allograft *in vivo*. Detailed mechanisms for the expansion of Treg upon adoptive transfer of Dex-induced MDSCs are not clear. Several mechanisms have been suggested to promote the expansion of Treg by MDSCs, including chemokine driven attraction of Treg (43, 44), and induced proliferation of natural Treg (45, 46). We identified a systemic expansion of Treg after adoptive transfer of MDSCs with an increased number of total Treg pool (data not shown), and *in vitro* culture of MDSCs with $CD4^+$ T cells can promote the expansion of Treg. Therefore, in the context of our model, MDSCs may directly expand the population of Treg through one or combined mechanisms mentioned above. Molecules responsible for the expansion of Treg by MDSCs includes iNOS signaling (1), CD274 (46), arginase (45), and IL-10 (22). In our study, no significant differences in CD274, Arg1 and TGF-β levels were found between MDSCs induced by GM-CSF or GM-CSF + Dex (**Figure 1C**, data not shown). Blockade of iNOS signaling in MDSCs with the use of iNOS KO mice also did not have an impact on the expansion of $CD4^+$ $Foxp3^+$ T cells *in vivo* (data not shown). The production of IL-10, which was shown to maintain Foxp3 expression in Treg (47) and also potentiated differentiation of human induced Treg (48), was significantly increased in Dex-induced MDSCs (data not shown). Future studies on the molecules responsible for the expansion of Treg are urged to fully address these questions.

In summary, we have shown that Dex treatment can enhance the expansion and immunosuppressive function of *in vitro*-generated MDSCs. Adoptive transfer of these Dex-induced MDSCs *in vitro* can also prolong cardiac allograft survival and favor the expansion of Treg *in vivo*, which may be a new approach of cellular therapy for organ transplantation.

ETHICS STATEMENT

All the animal experiments were carried out following the Guidelines for the Care and Use of Laboratory Animals of Nanjing University, and were approved by the ethical review committee of Nanjing University.

AUTHOR CONTRIBUTIONS

J-FD and Y-tD conceived and designed the experiments; X-FS, YZ, and KC performed the experiments; JD did the allogenic cardiac transplantation; X-FS and XK analyzed the data; J-FD and W-xG contributed reagents/materials/analysis tools; B-rL designed the experiments of Treg induction *in vitro*, X-FS wrote the paper.

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REVIEW
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Nanoparticles for the Induction of Antigen-Specific Immunological Tolerance

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Antigen-specific immune tolerance has been a long-standing goal for immunotherapy for the treatment of autoimmune diseases and allergies and for the prevention of allograft rejection and anti-drug antibodies directed against biologic therapies. Nanoparticles have emerged as powerful tools to initiate and modulate immune responses due to their inherent capacity to target antigen-presenting cells (APCs) and deliver coordinated signals that can elicit an antigen-specific immune response. A wide range of strategies have been described to create tolerogenic nanoparticles (tNPs) that fall into three broad categories. One strategy includes tNPs that provide antigen alone to harness natural tolerogenic processes and environments, such as presentation of antigen in the absence of costimulatory signals, oral tolerance, the tolerogenic environment of the liver, and apoptotic cell death. A second strategy includes tNPs that carry antigen and simultaneously target tolerogenic receptors, such as pro-tolerogenic cytokine receptors, aryl hydrocarbon receptor, FAS receptor, and the CD22 inhibitory receptor. A third strategy includes tNPs that carry a payload of tolerogenic pharmacological agents that can "lock" APCs into a developmental or metabolic state that favors tolerogenic presentation of antigens. These diverse strategies have led to the development of tNPs that are capable of inducing antigen-specific immunological tolerance, not just immunosuppression, in animal models. These novel tNP technologies herald a promising approach to specifically prevent and treat unwanted immune reactions in humans. The first tNP, SEL-212, a biodegradable synthetic vaccine particle encapsulating rapamycin, has reached the clinic and is currently in Phase 2 clinical trials.

Keywords: nanoparticles, immunological tolerance, rapamycin, tolerogenic dendritic cells, regulatory T cells

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INTRODUCTION

The central function of the immune system is the maintenance of immunological tolerance to self-components and innocuous exogenous antigens while eliminating malignant cells and dangerous pathogens. Immunological tolerance, defined as the absence of immunity to an antigen even in the presence of otherwise immunogenic stimuli, is achieved through a combination of processes that lead to the elimination or inactivation of immune cells specific for the antigen and the development of regulatory T cells (Tregs). The first and most impactful selection process, called central tolerance, eliminates lymphocytes recognizing self-antigens or leads to the differentiation of natural Tregs in the thymus. Autoreactive cells can escape this process and survive to join the repertoire of mature circulating lymphocytes. This pool of potentially dangerous cells can be further tolerized by

encountering their cognate antigen in absence of immunogenic signals leading to anergy or the induction of adaptive Tregs and the establishment of peripheral tolerance (1). For example, autoreactive lymphocytes specific for components of the nervous system can be identified in the circulation of animals and humans (2, 3). These cells remain dormant and checked by regulatory T and B cells. Similarly, most "foreign" gut-associated antigens (microbial or dietary) are well tolerated and do not trigger pathogenic immune responses. However, in presence of strong and persistent stimuli, lymphocytes specific for these antigens can break tolerance and launch attacks against self-components and innocuous antigens triggering disorders such as autoimmune diseases and food allergies, respectively.

Antigen-presenting cells (APCs), such as dendritic cells (DCs), are at the crossroads of immunity and tolerance (**Figure 1**). APCs sample and process antigens in the context of multiple complex cues from their environment. The pivotal signals allowing APCs to instruct lymphocytes to acquire the expression of costimulatory molecules and support the development of immunity have been categorized as "danger signals." Such signals include pathogen-associated molecular patterns (4), damage-associated molecular patterns (5), changes in the tissue metabolic state (6), inflammatory cytokines (7), and costimulatory-molecule ligands (8). Stimulation of APCs triggers a "maturation" program that includes activation of the NF kappa B (NF-κB) and mammalian target of rapamycin (mTOR) pathways and leads to metabolic changes and upregulation of costimulatory molecules, such as such as CD80, CD86, and CD40, and production of pro-inflammatory cytokines (9–11). By contrast, antigen presentation in the absence of such costimulatory signals results in anergy and tolerance (12, 13). APCs capable of tolerance induction include macrophages, B cells and DCs (14–17). Animals lacking DCs have a general failure in the establishment of self-tolerance, resulting in autoimmune conditions (18–22). Whether an immature or steady-state phenotype is required for DCs to induce tolerance is still a matter of debate. Recently the notion that tolerance is established by DCs that undergo incomplete maturation has been challenged by findings that tolerogenic DCs require transcriptional and epigenetic programs distinct from both steady-state (immature) and activated (mature) DCs (14, 19, 22–24). Furthermore, there is conflicting evidence about the phenotypic characteristics that define tolerogenic DCs induced by immunomodulatory drugs. For example, induction of tolerogenic capacities by treating DCs *in vitro* or *in vivo* with free or encapsulated rapamycin results in induced tolerogenic DCs (itDCs) of different phenotypes and maturation characteristics (e.g., expression of MHC-CLII and costimulatory molecules) (14, 25–30).

Regardless of the specifics of their phenotype, APCs constitute an ideal target to manipulate immune responses (**Figure 1**). Nanoparticles have unique properties that make them well suited to target APCs and deliver instructions that can modulate the nature of an antigen-specific immune response *in vivo* (31–35).

WHY NANOPARTICLES?

The immune system has evolved to capture and interrogate virus-like (nanosized) particles (36, 37). Such nanoparticulates are filtered out and accumulate in lymphoid organs, such lymph nodes and the spleen, and the liver. This scavenger task is performed by APCs that are adept at phagocytosing and eliminating debris in the extracellular environment. Synthetic nanoparticles of a wide array of materials in the range of 50 nm to 1 μm of size are readily phagocytosed by APCs (31, 32, 36–38). The display of multimerized antigen on nanoparticles has been shown to be inherently immunogenic, similar to particulate or aggregated antigen (36, 37, 39). Encapsulation or conjugation of antigens (both peptides and entire proteins) can lead to their presentation as a multimerized complex that has the potential to directly engage and cross-link of B cell receptors (BCRs), resulting in the activation of humoral immunity. Indeed, many particle-based vaccines exploit these principles (encapsulation and multimeric display) to induce protective humoral immunity (38).

To engineer nanocarriers for the induction of tolerance, we and others have use materials and components that provide tolerogenic signaling to APCs or harness natural tolerogenic processes to override the inherently immunogenic nature of antigen-bearing nanocarriers. The usage of synthetic tolerogenic nanoparticles (tNPs) confers several important advantages compared with other strategies to induce tolerance (**Table 1**). Nanoparticles can employ a wide range of materials that can be optimized for various functions and can carry a diverse payload of antigens and immunomodulators to deliver coordinated messages to the immune system.

This review will focus on nanoparticle approaches for the induction of antigen-specific immune tolerance. We define antigen-specific tolerance as the absence of immune response against an immunogenic target antigen, maintenance of tolerance after cessation of treatment, and retention of the ability to mount an immune response to an unrelated antigen. There have been three broad approaches to achieving antigen-specific immune tolerance with nanoparticles (**Figure 2**): (1) tNPs that provide antigen alone to harness natural tolerogenic processes or environments, (2) tNPs that provide antigen while targeting pro-tolerogenic receptors, and (3) tNPs that use pharmacological immunomodulators to force or "lock-in" a tolerogenic immune response against a target antigen. Nanoparticle delivery of immunomodulators, in the absence of a specific target antigen, for the treatment of autoimmune diseases and prevention of graft rejection is beyond the scope of this review, although it is notable that this approach has demonstrated durable disease modification in animal models (73–76). Similarly, nanoparticles that skew the immune response in an antigen-specific manner, such as Th1 polarizing nanoparticles for the treatment of Th2-mediated allergic diseases (77), are also not included in this review.

tNP PROPERTIES

Key attributes of nanoparticles affecting their function include material composition, size and charge. Materials for tNP manufacturing are diverse, and the choice depends on the desired function and compatibility with the payload. Three broad categories of materials include metals, liposomes, and synthetic and natural polymers. Metal and metal-oxide nanoparticles have

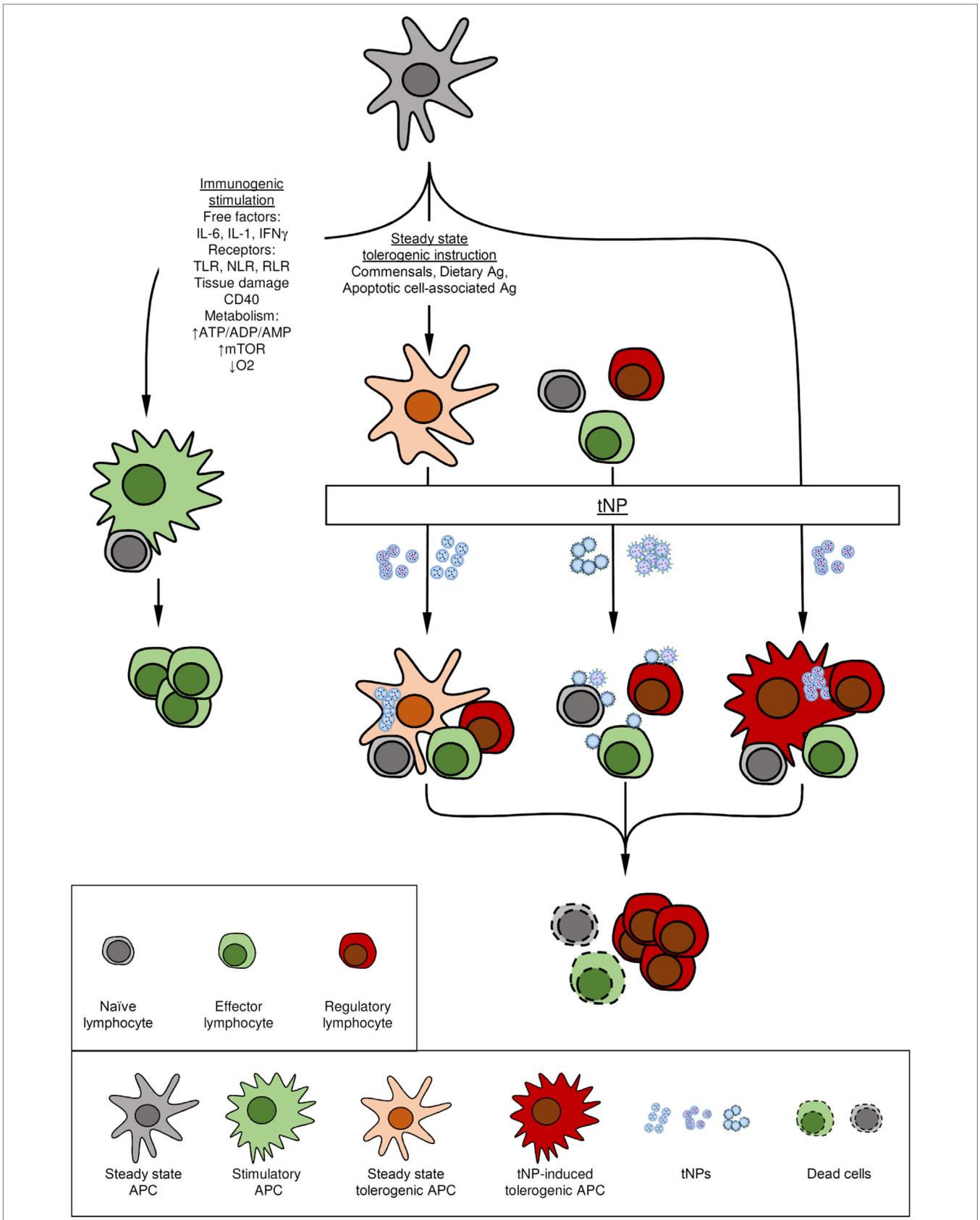

FIGURE 1 | Continued

FIGURE 1 | Mode of action of tolerogenic nanoparticles (tNPs). Antigen-presenting cells (APCs) play a major function in the immune system by integrating cues from the environment to promote immunity or tolerance. (A) Immunogenic stimuli such as cytokines, microbial components recognized by toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptor (RLR), and changes in the metabolic state of the tissue can activate APCs to induce immunity. (B) At steady-state immature APCs that capture self and innocuous antigens, such as those from commensal bacteria, present antigen in the absence of costimulation to induce or maintain tolerance. (C) Some tolerogenic NPs harness these natural tolerogenic processes by targeting tolerogenic delivery routes (oral tolerance), tolerogenic environments (liver), or mimicking apoptotic cells. (D) Other tNPs actively promote immune tolerance by employing pharmacological agents to induce tolerogenic dendritic cells. (E) Lymphocytes can also be targeted directly by tNPs that engage antigen-specific receptors in absence of costimulation or by targeting tolerogenic receptors. In all cases, tolerance is mediated by the preventing the activation of or eliminating antigen-specific cells both (naïve or effector) and/or the expansion of regulatory lymphocytes.

TABLE 1 | Tolerogenic nanoparticle (tNP) composition, mechanism, and characteristics.

tNP composition	Mechanism	Characteristics	Reference
Peptide–major histocompatibility complex (MHC) complexes on metal-oxide NPs or peptide–MHC complexes plus anti-Fas ligand antibody	Antigen presentation w/o costimulation on synthetic antigen-presenting cell. Anti-FAS ligand antibody delivers apoptotic signal	Direct action on effector T cells, but requires complex manufacturing. Restricted to peptide antigens (antigen selection risk). Non-biodegradable	(40–42)
Protein or DNA-encoded antigen in poly(lactic-*co*-glycolic acid) (PLGA) or chitosan NPs	Oral tolerance	Ease of delivery *via* oral route. However, poor history of translation for oral tolerance	(43–45)
Peptides conjugated to polystyrene, PLGA, or poly(maleic anhydride-alt-1-octadecene) nanoparticles	Mimic apoptotic cells; target tolerogenic niche *via* MARCO+ macrophages in spleen or liver sinusoidal cells	Simple composition, but restricted to peptides and i.v. dosing. Potential to be stimulatory in inflammatory setting	(46–52)
Antigen encapsulated in liposomes containing phosphatidylserine (PS)	Mimic apoptotic cells TAM? Scavenger receptor-mediated uptake by macrophages	PS-binding scavenger receptors trigger TAM? receptors and tolerogenic response	(53–57)
NPs encapsulating tolerogenic cytokines and antigen	Anti-inflammatory cytokines create a tolerogenic microenvironment?	Complex manufacturing. Potential to create autoreactive immune response to endogenous cytokines	(58–60)
Liposomes presenting antigen and CD22 ligand	Induce antigen-specific B cell tolerance and deletion	Direct action on specific B cells. CD22 ligand is a complex sugar that is difficult to manufacture. Requires protein antigen	(61, 62)
Gold particles presenting peptide antigen and aryl hydrocarbon agonist	Trigger aryl hydrocarbon receptor (AHR) pathway	Utilizes an immunomodulator (AHR agonist) to lock in tolerogenic response. Restricted to peptides? Non-biodegradable	(63, 64)
Liposomes containing peptide antigen and antigen	Inhibit NF kappa B (NF-κB) pathway	Utilizes an immunomodulator (NF-κB inhibitor) to lock in tolerogenic response. Works with protein antigens and s.c. or i.v. route	(65)
Polylactic acid/PLGA NPs containing rapamycin + antigen (encapsulated or free)	Induce tolerogenic dendritic cells by inhibition of mammalian target of rapamycin pathway	Utilizes an immunomodulator (rapamycin) to lock in tolerogenic response. Works with both protein and peptide antigens and s.c. or i.v. route. Human proof of clinical activity demonstrated	(30, 66–72)

been used for theranostics with capacity to carry antigens, targeting ligands, and immunomodulators on the particle surface (40, 41, 63, 64). These particles are very stable but typically require conjugation of the payload which may limit the application to certain molecules. A key disadvantage is that metal particles are not biodegradable, and hence accumulation may become a safety issue. Liposomes have been used in the clinic to deliver small molecule drugs and nucleic acids. Liposomes can incorporate various different phospholipids to create a membrane bilayer. The addition of cholesterol can alter the membrane fluidity of the lipid bilayer, which enables clustering of surface molecules upon interaction with target cells (62). In addition, phosphatidylserine (PS) lipids can be incorporated to target scavenger receptors involved in the phagocytosis of apoptotic cells (53). Liposomes can be adapted to incorporate various payloads with different physicochemical properties that get released after the liposome fuses with a cell membrane or after degradation in endosomes. The manufacturing of liposomes through a low shear extrusion method allows for encapsulation of proteins while minimizing the risk of denaturation. Molecules can be conjugated to the surface of liposomes but also hydrophilic molecules are amenable for encapsulation within the aqueous core of liposomes, while hydrophobic molecules can intercalate into the membrane bilayer. The release kinetics of the payload may be difficult to fine tune with liposomes. Various both natural and synthetic polymers have been used for tNP manufacturing, including polylactic acid (PLA), poly(lactic-*co*-glycolic acid) (PLGA), polystyrene, acetylated dextran, poly-L-lysine, polyacrylamide, and chitosan. The use of biodegradable polymers is preferred for safety. Biodegradable PLGA/PLA polymers have an excellent safety profile and have been used in various approved drugs and medical devices. PLGA/PLA tNPs are formulated by an evaporation emulsion method. Unlike liposomes, PLA/PLGA nanoparticles are solid particles in which the payload is embedded within the matrix. Hydrophobic molecules, such as rapamycin, dexamethasone, and vitamin D3, can be dissolved

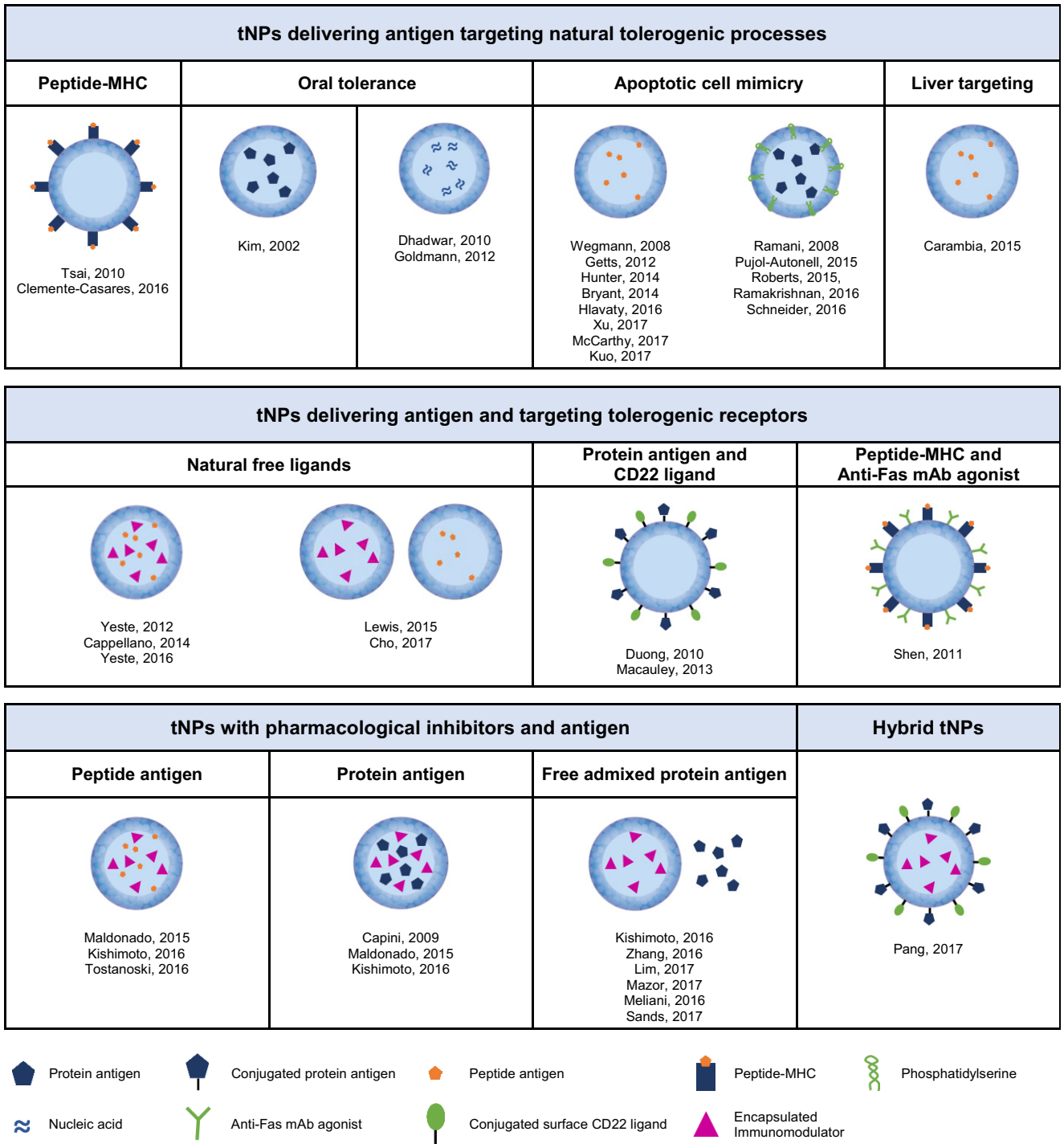

FIGURE 2 | Different types of tolerogenic nanoparticles according to their content and mechanism of action.

together with the polymer in a solvent and then emulsified with an aqueous phase-containing surfactants such as polyvinyl alcohol and pluronic (57, 59). Proteins and hydrophilic molecules can be incorporated through a double emulsion process (78). The release rate of the payload can be fine tuned by altering the glycolic acid to lactic acid ratio in PLGA, by changing the size of the polymer, and the use of excipients. The release rate can be further modified by conjugating molecules to the polymers which enables self-assembly of nanoparticles with the target molecules displayed on the surface or embedded within the matrix of the particles, as desired (79). The high shear process used to create nanoparticles may result in partial denaturation

of proteins. However, if the protein payload is an antigen, then denaturation is not an issue, as the antigen is targeted for degradation by APCs. Functional proteins can be attached to the surface of nanoparticles to preserve protein structure and function.

Nanoparticle surface charge has a strong influence on the immune responses. Typically, cationic nanoparticles lead to pro-inflammatory responses while anionic surfaces display reduced immunogenicity and longer circulation times. Anionic PLGA tNPs with zeta potential values in range of −40 to −70 mV have been shown to target a specific population of macrophages in the spleen expressing the scavenger receptor MARCO (48, 49, 51, 80, 81). In addition, many nanoparticle technologies use polyethylene glycol (PEG) to create a surface that is less prone to aggregation and opsonization by blood proteins, resulting in improved blood circulation properties (82, 83). Size can also affect circulation time and biodistribution. Particles smaller than 6 nm drain to the blood whereas particles larger than 9 nm tend to preferentially drain to lymphatics (84). Nanoparticles in the range of 20–100 nm have been shown to accumulate in the liver in liver sinusoidal endothelial cells (LSECs) or macrophages upon i.v. injection while after s.c. injections bigger particles have a tendency to become trapped in the extracellular environment and require active transport by phagocytes to reach the draining lymph nodes (85–88). Particles from 100 to 200 nm can traffic to both the spleen and liver when injected i.v. and circulate through the lymph after s.c. injections to be taken up by lymph sinus DCs that accumulate in draining lymph nodes (88, 89). Bigger particles from 200 nm to 5 μm accumulate mostly in the spleen when injected i.v. and require active cellular transport to reach lymph nodes when injected s.c. (50, 66).

Ultimately, the choice of materials comes down to optimizing compatibility with the desired payload and activity. Within each class of material, there is a considerable range of options that can be used to optimize nanoparticles for specific payloads (e.g., immunomodulators) and activity (e.g., release rates). For example, there are different types of metals that can be used, a range of lipids that impart different properties to liposomes, and different types of polymer chemistries. Unfortunately, optimization is largely an empirical exercise. Therefore, it is important to have robust screens to optimize the features that are most desirable. While NF-κB inhibitors (65) and aryl hydrocarbon receptor (AHR) agonists (63, 64) have been demonstrated to be tolerogenic in liposomes and on gold particles, respectively, in our hands, these immunomodulators were not active in pilot PLGA nanoparticle formulations. It is possible that further optimization would be required to create similarly active particles with PLGA. However, certain nanoparticle materials may naturally lend themselves to be more compatible with certain types of immunomodulators.

If more than one payload is needed (e.g., antigen and immunomodulator), it also important to optimize the ratio of these two components. This can be difficult as one component may affect the encapsulation efficiency of the second component, thus it may be difficult to create a series of particles in which the load of the first component is held constant while varying the load of the second component. One strategy to work around this issue is to encapsulate the two components into separate nanoparticle formulations and admix different ratios of the two particles (66). This strategy is effective if the biodistribution of the two particles is sufficiently similar. Since a therapeutic dose of nanoparticles may involve the injection of billions of nanoparticles, APCs will endocytose hundreds of nanoparticles. However, one must keep in mind that B cells specific for the target antigen may selectively take up nanoparticles containing antigen. Once an optimal ratio is determined, then a single particle formulation containing both components in the appropriate ratio can be created and tested. There is, however, a risk that the optimal ratio for mice may be different in humans. Thus, it is worth considering to develop a two particle formulation for initial human clinical trials, so that different ratios can be evaluated.

TOLEROGENIC NPs THAT PROVIDE ANTIGEN ALONE TO HARNESS NATURAL TOLEROGENIC PROCESSES OR ENVIRONMENTS

Immune tolerance is the homeostatic, default pathway for antigen presented in the absence of costimulation in steady-state, non-inflammatory environments (14, 20, 90). The immune system has evolved to distinguish between cells that undergo natural cell death, or apoptosis, versus necrotic cell death due to injury or infection. There are natural tolerogenic processes that not only maintain tolerance to self-antigens but also enable induction and maintenance of tolerance to innocuous food antigens and commensal bacterial antigens. Providing antigen in the context of these natural tolerogenic processes and environments is one strategy to induce antigen-specific tolerance.

Peptide Antigens Presented on MHC-Bearing tNPs

T cells recognize antigenic peptides presented in the context of major histocompatibility complex (MHC) class I (CD8 T cells) or MHC class II (CD4 T cells) and require a costimulatory second signal for activation of effector T cells. In the absence of costimulation, T cells become anergic, undergo apoptosis or differentiate into regulatory T cells (12, 13). Santamaria and colleagues created "synthetic APCs" by coating iron oxide nanoparticles with specific peptide–major histocompatibility class 1 complexes (pMHC-CLI). These tNPs present antigen in the absence of costimulatory molecules resulting in suppression of autoreactive CD8+ T cells and their conversion to a regulatory, anergic phenotype that controlled pathogenic responses by a mechanism dependent on IFNγ secretion, indoleamine 2,3-dioxygenase, and perforin expression (41). Importantly, this tolerance was antigen specific and dominant as transfer of CD8+ T cells from treated mice into a naïve prediabetic NOD animal conferred protection from development of type 1 diabetes (T1D).

This approach has also been extended to MHC class II molecules loaded with disease-relevant peptides (pMHC-CLII). These tNPs were efficacious in animal models of T1D, experimental autoimmune encephalomyelitis (EAE) and arthritis, but they worked through a different mechanism than pMHC-CLI-loaded

tNPs (40). Treatment with pMHC-CLII-presenting tNPs led to the differentiation of IL-10-producing Tr1 regulatory cells and regulatory B cells that were capable of transferring tolerance to untreated animals, even if the cells were antigen-experienced, suggesting that this approach could lead to reversal of memory responses.

While this approach is elegant, one of the hurdles for clinical development is the identification and validation of the relevant MHC alleles and peptides in the diverse human population. T1D and celiac disease may be the most amenable diseases for this approach, as there are strong disease associations with MHC-DQ/DR alleles. Other diseases may require GMP manufacturing of multiple tNPs bearing different MHC alleles to treat a heterogenous population.

Harnessing Oral Tolerance

Delivery of antigen through the oral route has been shown to be tolerogenic, presumably through a mechanism that is similar to the tolerogenic response observed in all healthy individuals to dietary and gut flora antigens. However, current approaches to induce tolerance orally require chronic and frequent treatments (91). Kim et al. were among the first to load nanoparticles with antigen alone in the context of a model of collagen-induced arthritis (CIA). The authors showed that PLGA particles loaded with type II collagen (CII) provided orally 14 days before immunization with CII prevented CIA, antigen-induced T cell proliferation, anti-CIIA antibodies in a dose-dependent manner. The Peyer's patches of treated animals had an increased TGFβ/TNFα ratio, suggesting an active anti-inflammatory program in response to the antigen (43). Dhadwar et al. showed that repeated weekly administrations of chitosan nanoparticles containing DNA encoding coagulation factor VIII (FVIII) provides sustainable FVIII activity in hemophilia A mice while avoiding the induction of inhibitory and non-neutralizing anti-FVIII antibodies (44). Goldmann et al. investigated a similar approach with ovalbumin (OVA)-encoding DNA encapsulated in chitosan tNP and showed suppressed OVA-specific delayed-type hypersensitivity (DTH) and anti-OVA antibody responses and transferable tolerance mediated by CD4+ CD25+ T cells (45).

While oral tolerance has been shown to be effective in mice, human clinical trials of oral tolerance have been largely disappointing. It is also not clear if non-viral oral delivery of DNA-bearing nanoparticles in humans would result in sufficient and sustained levels of antigen expression to induce immune tolerance.

Harnessing Apoptotic Cell Death

Cells that undergo necrotic cell death, due to tissue damage or infection, induce an immunogenic response, while cells that undergo apoptosis, or programmed cell death, generally induce a tolerogenic response (92, 93). Coupling antigens to spleen cells through ethylenecarbodiimide (ECDI) fixation has been shown to induce their apoptosis and treatments with these dead cell-peptide conjugates confers tolerance to the antigen in many preclinical models. This approach has been used to treat various diseases, including animal models of EAE and T1D (94, 95). A similar strategy has been employed to target disease-relevant antigens to erythrocytes *in vivo*. Erythrocyte cell death led to immunologic tolerance to the bound antigen and protection in a model of autoimmune T1D (96). The adaptation of this approach to nanoparticles has involved delivery of peptide antigens coupled to dendrimers and to polystyrene and PLGA carriers. Treatment with pathogenic peptides conjugated to dendrimers protected animals from developing EAE with a concomitant reduction of effector T cells in the CNS (46). A similar approach using 500 nm carboxylated polystyrene beads with a mixture of immunodominant HLA-A*02:01-restricted epitopes was used to treat HHD II mice (β2mKO/HLA-A*0201 transgenic mouse) and inhibit diabetogenic human cytotoxic T cell (CTL) responses in a Treg-dependent manner (52). Negatively charged 500 nm PLGA nanoparticles delivered intravenously target splenic macrophages that express the scavenger receptor MARCO (47). This approach has shown to be efficacious in preventing and treating autoimmune processes in relapsing–remitting EAE and T1D and in preventing graft rejection in bone marrow transplantation and allogenic pancreatic islet transplantation (47–49, 51, 80, 81, 94, 95). Tolerance induction was demonstrated by challenging treated animals with the antigen after disease resolution or the prolonged survival of grafts (47, 48, 51) and the elimination of pathogenic effector T cells by induction of apoptosis and anergy (94, 95). The precise mechanism for the induction of tolerance by negatively charged tNPs remains to be elucidated. Recently Kuo et al. described that pro-inflammatory transcription factors NF-κB and STAT1 are triggered in macrophages and DCs when incubated with this type of nanoparticle. However, the cells had a decreased capacity for presenting antigen, displayed a restricted costimulatory-molecule phenotype with low expression of CD86, CD80, and CD40 and showed upregulation of STAT3, IL-10, and sustained PD-L1 expression, a profile associated with anti-inflammatory functions (80). Interestingly this phenotype was observed in both macrophages and DCs, while tolerance induced by negatively charged tNPs has been described to be dependent on MARCO+ macrophages only (47). It is not clear how negatively charged nanoparticles mimic apoptotic cells, or whether they recapitulate the full tolerogenic phenotype of apoptotic cells. It appears that the use of charged nanoparticles to induce tolerance works best with peptide antigens, while ECDI-fixed splenocytes and erythrocytes undergoing cell death can confer tolerance to either peptide or protein antigens. It is possible that the inherently immunogenic properties of protein antigen displayed in a multimeric fashion on NPs cannot be overcome with this approach. Interestingly, the survival of allogenic pancreatic islets graft in mice treated with poly(lactide-*co*-glycolide) particles containing alloantigen was synergistic with low dose free rapamycin, suggesting that this approach could be substantially improved by the addition of an immunosuppressant (49).

Another approach to mimic apoptotic cells is to cloak liposomes with Phosphatidylserine (PS). PS is a phospholipid forming part of the cell membrane that is translocated from the cytosolic (inner) to the extracellular (outer) membrane of cells undergoing apoptosis. Macrophages express various PS-specific scavenger receptors, such as Tyro3, Axl, and Mertk (collectively referred to as TAM receptors), TIM-3 and SCARF-1 that trigger the phagocytosis of dying cells and promote induction of a

tolerogenic phenotype, such as the increase in IL-10 and TGFβ secretion, and a decrease in NF-κB signaling and TNFα, IL-1β, and IL-12 secretion (97). Encapsulating coagulation FVIII in PS-bearing liposomes was effective to prevent the formation of inhibitory anti-FVIII antibodies in hemophilia A animals even when animals were challenged with FVIII alone (53, 57, 98, 99). A similar approach was demonstrated with alpha-glucosidase (GAA) in a mouse model of Pompe disease. Administration of GAA-containing PS liposomes provided therapeutically active enzyme while preventing the formation of inhibitory antibodies (56). In an autoimmune setting, PS liposomes loaded with disease-relevant peptides were protective in the NOD animal model of T1D (54) and PLGA nanoparticles displaying PS and containing peptide autoantigens (from myelin oligodendrocyte protein, $MOG_{35–55}$) were also efficacious in an acute model of EAE (in B6 mice) (55).

Harnessing the Tolerogenic Environment of the Liver

The liver is considered a tolerogenic organ due to its unique function in filtering antigens from blood delivered from the gastrointestinal tract *via* the hepatic portal veins (100, 101). The liver is constantly bathed in food antigens and commensal bacterial products, "foreign" products to which immunological tolerance must be induced and maintained in healthy organisms. Carambia et al. (50) have shown that peptide-coupled poly(maleic anhydride-alt-1-octadecene)-coated nanoparticles injected i.v. protected mice in a model of EAE in a Treg-dependent manner. Microscopy studies showed selective uptake of these nanoparticles by LSECs.

TOLEROGENIC NPs THAT PROVIDE ANTIGEN WHILE TARGETING TOLEROGENIC RECEPTORS

One of the potential concerns about delivering nanoparticles containing only antigen is that in an inflammatory microenvironment, these tNPs could inadvertently provoke a stimulatory immune response and exacerbate an autoimmune condition. One strategy to mitigate this risk is to create tNPs that deliver antigen while simultaneously targeting tolerogenic receptors.

Harnessing Cytokine Mediators of Immunological Tolerance

One strategy is to create a tolerogenic environment by co-delivery of antigen with nanoparticle-encapsulated anti-inflammatory cytokines and soluble mediators. Encapsulated $MOG_{35–55}$ and rIL-10 ameliorated the course of EAE induced with $MOG_{35–55}$ in C57BL/6 mice (58). Furthermore, two groups developed a system of multiple microparticles with different functionalities engineered to be phagocytosed and release their cargo in the intracellular space or avoid phagocytosis and release anti-inflammatory cytokines in their environment. This dual system allowed for the release of antigen and vitamin D3 inside APCs and TGFβ and GM-CSF extracellularly. Although tolerance induction was not demonstrated, treatments with these particles showed the immunoregulatory capacity of encapsulated cytokines by preventing T1D in NOD animals (59) and EAE in $MOG_{35–55}$–immunized animals (60) consistent with a general suppressed phenotype of CD4+ T cells and a tolerogenic phenotypes on APCs. A hurdle for clinical development is the requirement to produce one or more GMP cytokines.

Harnessing AHR Agonists

The AHR is a ligand-activated transcription factor that controls the differentiation of Foxp3+ and IL-10+ Tregs and Th17 cells. Quintana and colleagues have shown that an endogenous AHR ligand, 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), co-delivered with myelin peptide $MOG_{35–55}$-loaded on gold nanoparticles promote the generation of Tregs *in vitro* and *in vivo* that were capable of transferring tolerance to naïve animals (63). This approach was also efficacious with protein antigen, as ITE and proinsulin-loaded tNPs suppressed autoimmune diabetes in NOD animals. Interestingly, treatment with these particles induced a Socs2-dependent itDC phenotype characterized the inhibition of NF-κB signaling, a decreased ability to active Teff cells and an increased differentiation of Foxp3+ Treg cells (64).

Targeting the Fas Receptor to Kill Antigen-Specific Effector Cells

The Fas receptor mediates programmed cell death. Shen et al. created artificial APCs with latex beads coated with pMHC-CLI complexes and a monoclonal antibody directed against Fas receptor, which caused the deletion of antigen-specific CTLs in an animal model of skin graft (42). A potential hurdle for clinical development is the cost of producing GMP manufactured monoclonal antibodies.

Targeting B Cell-Specific Tolerance through CD22

B cells play a unique role in the immune system by serving as both APCs and antigen-specific effector cells. The BCR, a transmembrane splice variant of an antibody, can directly bind to its cognate antigen and trigger B cell activation. To prevent activation to autoantigens, B cells express inhibitory co-receptors, such as CD22, a member of the SIGLEC family of lectins that binds to sialic acid-bearing glycoproteins and glycolipids. Co-localization of CD22 with the BCR results in the recruitment of phosphatases that inhibit BCR signaling and result in B cell deletion. The Paulson group developed liposomal nanoparticles, called SIGLEC-engaging tolerance-inducing antigenic liposomes (STALs) displaying both antigen and CD22 glycan ligands on their surface that induce apoptosis in mouse and human B cells. Animals treated with STALs did not develop antibody responses to T cell-independent antigens, such as nitrophenol, or T cell-dependent protein antigens, such as coagulation FVIII, even after repeated immunogenic challenges (61, 62). It is notable that this approach is compatible with the use of protein antigens, as the inhibitory signal delivered by CD22 co-engagement is sufficient to override the inherent immunogenicity of a protein-bearing nanocarrier. One potential hurdle for clinical development is the difficulty and cost of synthesizing CD22 glycan ligands.

TOLEROGENIC NPs THAT HARNESS TOLEROGENIC PHARMACOLOGICAL AGENTS

Recently, a number of researchers have investigated pharmacological agents capable of inducing tolerogenic DCs (102–104). The potential advantage of pharmacological mediators of tolerance is the potential ability to "lock-in" a tolerogenic phenotype even in the face of an inflammatory microenvironment. While autologous tolerogenic DCs induced *ex vivo* could be used therapeutically, such individualized cell therapy would be costly and difficult to scale. However, nanoparticles represent an ideal "off-the-shelf" vehicle to deliver a payload of both target antigen and tolerogenic drug to induce endogenous tolerogenic DCs *in vivo*. To date, pharmacological agents targeting at least two different signaling pathways have been used in tNPs to induce antigen-specific tolerance *in vivo*.

NF-κB Inhibitors

NF kappa B is a master regulator of a broad array of genes controlling inflammation and cell survival. Thomas and colleagues have demonstrated that co-delivery of antigen with various NF-κB inhibitors, such as curcumin, quercetin, and Bay11-07082, in liposomes suppressed inflammatory arthritis in an antigen-specific manner (65). The liposomes accumulated in lymph nodes and spleen following i.v. injection and were taken up by MHC class II+ APCs resulting in inhibition of NF-κB activation. Mice treated with liposomes showed induction of Ag-specific Foxp3+ regulatory T cells, which conferred protection when adoptively transferred into naïve animals. Depletion of Tregs with anti-CD25 antibodies abrogated the tolerogenic activity of the tNPs.

mTOR Inhibitors

Mammalian target of rapamycin is a conserved serine/threonine kinase that integrates environmental signals to regulate cell metabolism and survival. Rapamycin is a natural product derived from *Streptomyces hygroscopicus*, which binds to the FK506-binding protein to form a complex that acts as an allosteric inhibitor of the mTOR complex-1 pathway. Rapamycin was found to have potent immunosuppressive activity based on its ability to inhibit T cell proliferation and is approved for the prophylaxis of renal allograft rejection. Importantly rapamycin treatment has been shown to promote Treg expansion and differentiation (27, 105, 106). In addition to its direct effects on T cells, Thomson and colleagues have demonstrated that *in vitro* treatment of DC induces a tolerogenic phenotype (25, 27).

We screened a large number of immunomodulators for compatibility with biodegradable PLA and PLGA nanoparticles and found that rapamycin-loaded nanoparticles showed potent tolerogenic activity *in vivo*. We and others have shown that tNPs containing rapamycin induced durable antigen-specific immune tolerance when coadministered with various encapsulated or free protein and peptide antigens. These tNPs were selectively taken up by APCs in lymphoid organs (66) and demonstrated efficacy when coadministered with antigen by i.v., s.c., or direct intranodal injection (66, 69). The tNPs were shown to generated itDCs and Foxp3+CD4+ T cells *in vivo* and inhibit CD4+ and CD8+ T cell effector cell activation (30, 66). Moreover, weekly doses of tNPs encapsulating rapamycin, but not daily doses of free rapamycin, were effective in inducing immune tolerance (30). Indeed, a single dose of tNPs co-encapsulating rapamycin and antigen inhibited antigen-specific T cell expansion while increasing the proportion of Foxp3+ T cells, while the equivalent doses of encapsulated antigen with free rapamycin had the opposite effect (66). This difference may be attributed to the selective targeting of tNPs to APCs in the draining lymph nodes. Importantly, immune tolerance induced by tNPs encapsulating rapamycin was effective even when coadministered with a potent TLR7/8 agonist and was maintained in animals challenged with antigen coadministered with TLR agonists or emulsified in complete Freund's adjuvant. Tolerogenic NPs containing co-encapsulated antigen and rapamycin were effective in preventing T cell-mediated pathologies such as DTH reactions and EAE. In addition, therapeutic treatment at the peak of disease was effective in reversing paralysis in a model of EAE (66, 69). Tolerance induced by tNPs encapsulating rapamycin could be transferred to naïve animals (107).

In addition, rapamycin-containing tNPs inhibited B cell activation and differentiation into effector cells, germinal center formation and antibody production. These rapamycin-containing tNPs were effective in preventing IgE-mediated anaphylaxis in models of allergy, IgG-mediated anaphylaxis associated with repeated intravenous challenges with antigen, and the formation of anti-drug antibodies (ADAs) to a wide range of biologic drugs. Coadministration of tNPs containing rapamycin with free biologic drugs was effective in preventing ADAs against coagulation FVIII (Advate®) in a model of hemophilia A (66, 67); human TNFα-blocking antibody adalimumab (Humira®) in a model of inflammatory arthritis (30), acid-α-glucosidase (Lumizyme®) in a model of Pompe disease (70), recombinant immunotoxin in a model of mesothelioma (71), adeno-associated virus gene therapy vectors (68) and pegylated uricase (pegsiticase) in uricase-deficient mice and non-human primates (30). Currently the combination of tNP-rapamycin and pegsiticase (SEL-212) is in Phase 2 clinical trials (NCT02959918) in patients with symptomatic gout and hyperuricemia (see Human Translation).

HYBRID tNPs

Strategies that employ tNPs that harness natural tolerogenic mechanisms and those that incorporate a pharmacological mediator of tolerance may have synergistic effects. Recently Paulson and colleagues added low doses of rapamycin to their STALs liposomes that present antigen in context with a CD22 ligand (108). While CD22 ligand co-localized with antigen would directly inhibit antigen-specific B cell activation, it would not prevent T cell activation by DCs and macrophages that also take up the STALs particles. Preliminary data indicate that the addition of low dose rapamycin enhanced the tolerogenic response, presumably by mitigating antigen-specific T cell activation.

HUMAN TRANSLATION

Currently, the treatment of autoimmune diseases requires lifelong use of general immunosuppressants or immunomodulators that may target-specific pathways (e.g., TNF-α) but are not antigen-specific. A long-standing goal for immunotherapy is the development of antigen-specific therapies that leave the rest of the immune system intact and that can arrest or even reverse disease pathology. Clinical translation of other (non-nanoparticle-based) strategies to induce antigen-specific tolerance induction has been challenging and largely disappointing. The immune system is a complex network of cells, organs, and soluble factors that must integrate multiple environmental cues to determine how to respond to a given antigen. Nanoparticles are ideal vehicles to mediate antigen-specific immune modulation, as they can be engineered to provide multiple coordinated signals to shape the immune response. For example, nanoparticles have been developed for stimulatory vaccines by incorporating antigen and TLR agonists or other pro-inflammatory adjuvants. Creating tolerogenic or "inverse" vaccines using nanoparticles that harness natural tolerogenic mechanisms or employ tolerogenic pharmacological agents is an attractive concept. The preclinical data demonstrating induction of antigen-specific tolerance using a wide variety of nanoparticle materials and strategies is compelling. However, clinical translation remains a key hurdle for the field.

It is worth considering why clinical translation in immune tolerance has been so vexing. There are several factors to consider. (1) Animals models of autoimmunity are poorly predictive of human disease. In addition to obvious differences in the immune system, genetic diversity, lifespan, and environmental factors between humans and laboratory mice, there are a number of limitations of mouse models specific to autoimmune diseases. For example, many mouse models of autoimmunity are homogenous acute models that often, as in the case of EAE, use a single immunization with a single antigen to trigger pathology while most autoimmune disorders in humans are chronic and heterogenous diseases that develop over many years. (2) Antigen uncertainty. For many human autoimmune diseases, there are usually multiple candidate antigens with epitope spreading that occurs with disease progression. Moreover, the pathogenic antigens may vary from patient to patient. This antigen selection risk is compounded for peptide-based immunotherapies, as dominant peptide epitopes may vary widely among different patients due to heterogeneity in MHC alleles and T cell receptor repertoire. (3) Requirement for therapeutic activity in established disease. Modifying a memory immune response is considerably more challenging than affecting a naïve response. For example, there are many highly effective prophylactic vaccines dating back to 1796 with Jenner's small pox vaccine; however, there is only one approved therapeutic vaccine, Provenge®, a modestly effective DC vaccine for prostate cancer. In mouse models of autoimmune disease, it is difficult to study true therapeutic activity in the setting of well-established disease involving memory T cell populations. Thus, it is challenging to assess whether the failure of clinical translation of immune tolerance strategies is due to non-predictive animal models, incorrect antigen selection, or short-comings of the therapeutic strategy.

To mitigate some of these challenges in assessing the clinical efficacy of rapamycin-containing tNPs, we have chosen to focus on the mitigation of ADAs to biologic therapies (30). ADAs are a common cause for biologic treatment failure and hypersensitivity reactions. Using this strategy confers several significant advantages as (1) the animal models are simple immunization models, and the clinical proof-of-concept is relatively straightforward with a well-established and easily measured biomarker readout (ADA titers), (2) the antigen is unequivocally known, as it is the biologic drug itself, and (3) the tolerizing therapy can be administered prophylactically, as it is known when the patient receives the drug. Our lead clinical program, SEL-212, is the first immune tolerizing nanoparticle technology to reach the clinic. SEL-212 is a combination therapy consisting of rapamycin-containing tNPs coadministered with pegylated uricase, a uric acid metabolizing enzyme, for the treatment of severe, chronic gout. Preclinical studies demonstrated the ability of these tNPs to prevent the formation of ADAs in uricase-deficient hyperuricemic mice enabling the enzyme to achieve sustained control of serum uric acid levels (30). Similar effects on ADA prevention were obtained in rats and non-human primates (30). A single ascending dose Phase 1 clinical trial of SEL-212 (NCT02648269) in patients with hyperuricemia showed that the pegylated uricase is highly immunogenic in humans even after a single dose of enzyme. The addition of tNPs showed a dose-dependent inhibition of anti-uricase antibody formation resulting in sustained reduction of serum uric acid levels (72). An ongoing Phase 2 study (NCT02959918) will assess the ability of multiple doses of SEL-212 to inhibit the formation of ADAs with in patients with symptomatic gout and hyperuricemia.

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REVIEW
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Regulatory T-Cells: Potential Regulator of Tissue Repair and Regeneration

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The identification of stem cells and growth factors as well as the development of biomaterials hold great promise for regenerative medicine applications. However, the therapeutic efficacy of regenerative therapies can be greatly influenced by the host immune system, which plays a pivotal role during tissue repair and regeneration. Therefore, understanding how the immune system modulates tissue healing is critical to design efficient regenerative strategies. While the innate immune system is well known to be involved in the tissue healing process, the adaptive immune system has recently emerged as a key player. T-cells, in particular, regulatory T-cells (Treg), have been shown to promote repair and regeneration of various organ systems. In this review, we discuss the mechanisms by which Treg participate in the repair and regeneration of skeletal and heart muscle, skin, lung, bone, and the central nervous system.

Keywords: CD4$^+$ regulatory T-cells, tissue repair and regeneration, stem cells, macrophages, heart regeneration

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INTRODUCTION

The global number of individuals suffering from organ dysfunction as a result of acute injuries, chronic disorders, or aging has been on the rise and thus inadvertently places a high demand for organ transplantation. However, organ and tissue transplantation is obviously limited by the shortage of donors and side effects associated with the use of immunosuppressants (1), placing stress upon current methodology and creating a need for an alternative therapeutic avenue. By virtue of its self-renewal properties and capability in differentiating into multiple cell types, recent advances in human pluripotent stem cell research has offered a literally unlimited amount and varieties of therapeutic cells for transplantation (2, 3). Nevertheless, there is a lack of clinical evidence showing their long-term engraftment following transplantation possibly due to poor cell survival and chronic immune rejection (4, 5). Moreover, regenerative therapies stimulating endogenous regeneration such as growth factor-based strategies have shown mixed results in the clinic due to safety concerns and cost-effectiveness (6, 7). Therefore, it is necessary to find new ways to improve regenerative strategies and one of them is to control and utilize the host immune system. Nevertheless, in order to design immune-centric regenerative therapies, it is imperative to understand how the various immune components modulate tissue repair and regeneration.

Since decades, the immune system is well known to be implied in tissue repair and regeneration. For instance, inflammation following injury greatly contributes to tissue repair and scar formation, while excessive inflammation led by immune cells causes pathological fibrosis that debilitates tissue function and may lead to organ failure. Immune-mediated tissue healing processes are complex, yet, highly orchestrated. After injury, invading pathogens, necrotic debris, the clotting reaction, and

tissue-resident immune cells trigger an inflammatory response, which result in the recruitment of various immune cells. The activity of immune cells during wound healing can be separated in three phases (8): First, pro-inflammatory cells are recruited to the site of injury for host defense and phagocytosis of necrotic tissues. Second, the pro-inflammatory response is dampened *via* immune cells such as anti-inflammatory macrophages, while immune cells also directly participate in stimulating angiogenesis, myofibroblast activation, and tissue progenitor cell proliferation. Last, most immune cells exit the site of injury or are eliminated by apoptosis and the tissue homeostasis is restored. Nonetheless, the role of the various immune cells and their subsets as well as the mechanisms by which they regulate tissue healing remain largely elusive. It is, therefore, imperative to understand how tissue healing is controlled by the immune system and harnessing the endogenous regenerative capacity has recently become an active area of research.

An interesting observation supporting the critical role of immunity in regeneration (as opposed to tissue repair and scarring) comes from the evolution of the immune system among species and during development. Compared to lower vertebrates such as amphibians and teleost fishes that are capable of completely regenerating many body parts, mammals have a limited regenerative potential. To explain this difference, it has been postulated that the loss of regenerative capacity in mammals is in part associated with maturation of their immune system compared to lower vertebrates (9, 10). The immune system also changes during development and throughout life. For example, some organs such as the mammalian heart is notorious for not being able to regenerate and the necrotic cardiac muscles are replaced by dysfunctional scar tissues after injury. However, accumulating evidence shows that the neonatal hearts of mammals including humans have a transient regenerative capacity compared to adults (11–13). Indeed, the mammalian adaptive immune system is relatively immature after birth, which coincides with the period of neonatal regeneration. In contrast to adults, neonates do not mount a robust fibrotic but a more angiogenic response that facilitates tissue regeneration after injury (10). Therefore, since immune cells regulate both fibrosis and angiogenesis during tissue healing, targeting the immune system to promote neoangiogenesis with minimal fibrosis would be an interesting approach to stimulate regeneration. Therefore, understanding how immunity regulates tissue fibrosis and neoangiogenesis would shed light on the development of potential therapeutics targeting endogenous tissue regeneration. During the last decade, innate immunity, in particular, macrophages and their various polarization states, has been considered as a central regulator of the tissue healing process. However, recent evidences suggest that the adaptive immune system is also a critical actor. In this review, we focus on the role of regulatory T-cells (Treg).

OVERVIEW OF THE IMMUNE FUNCTIONS OF Treg DURING TISSUE HEALING

Treg are required for maintenance of self-tolerance, preventing excessive inflammation and autoimmune diseases. The most reliable cell-specific marker of Treg is Forkhead box P3 (FOXP3), which is essential for the maturation and function of Treg. Congenital deficiency in Treg, due to mutation of the *Foxp3* gene, causes fatal autoimmunity in mice, the scurfy phenotype, and enteropathy, X-linked (IPEX) syndrome in human (14, 15). Treg are normally present in lymphoid organs but have been shown to accumulate in damaged tissues to some extent. Long recognized as potent suppressors of the immune system, Treg have been recently rediscovered as indirect and direct regulators of tissue healing, while the mechanisms are still largely unknown (16–18).

Uncontrolled inflammation after tissue injury can lead to impaired healing and tissue remodeling. In many tissues, Treg are recruited to the damaged site to facilitate inflammation resolution and to regulate immunity after injury (19). For instance, Treg can indirectly modulate regeneration by controlling neutrophils (20–22), inducing macrophage polarization (23, 24), and regulating helper T-cells (22, 25) (**Figure 1**) Moreover, Treg have been shown to directly facilitate regeneration *via* activating progenitor cells locally (16, 17).

Treg INTERACT WITH INNATE IMMUNE CELLS TO CONTROL INFLAMMATION AFTER TISSUE INJURY

Treg are able to control the functions of neutrophils and macrophages, which have been widely shown to be involved in the tissue healing process. Neutrophils are among the first leukocytes recruited to the injury site, and they directly modulate tissue healing either positively or negatively. For instance, after skeletal muscle injury, it has been demonstrated that neutrophils impair restoration of muscle structures and function through the release of hypochlorous acid, NAPDH oxidase, and other cytokines (26, 27). A negative role of neutrophils has also been demonstrated in a lung ischemia-reperfusion model, where neutrophils enhance the injury (28). However, in an inflammatory lung disease model, mice treated with intratracheal LPS, which induces neutrophil transmigration show activated β-catenin signaling in lung epithelial cells, triggering repair of the lung epithelium (29). Therefore, it is likely that neutrophils modulate tissue healing in a context-specific manner.

Treg have shown ability to modulate tissue healing *via* controlling neutrophil behavior. For instance, an *in vitro* study has demonstrated that activated Treg promote neutrophils to secrete anti-inflammatory molecules including IL-10 and TGF-β, heme oxygenase-1, and indoleamine 2,3-dioxygenase (IDO). This is also preceded by inhibition of neutrophil's IL-6 production, suggesting that Treg can modulate inflammation through inhibition of neutrophil activity (30). Concomitantly, Treg have been shown to induce neutrophil apoptosis and death both *in vitro* and *in vivo* (21, 31). For example, in an acute lung injury model, Treg mediate resolution of lung injury *via* TGF-β-induced neutrophil apoptosis (21). In addition, Treg can modulate neutrophil infiltration to the site of injury. For instance, deletion of Treg leads to increased infiltration of neutrophils after cardiac injury and subsequently results in impaired healing (20, 22). In a model of kidney ischemia reperfusion injury, Treg suppress infiltration of neutrophils and attenuate kidney injury *via* IL-10 secretion

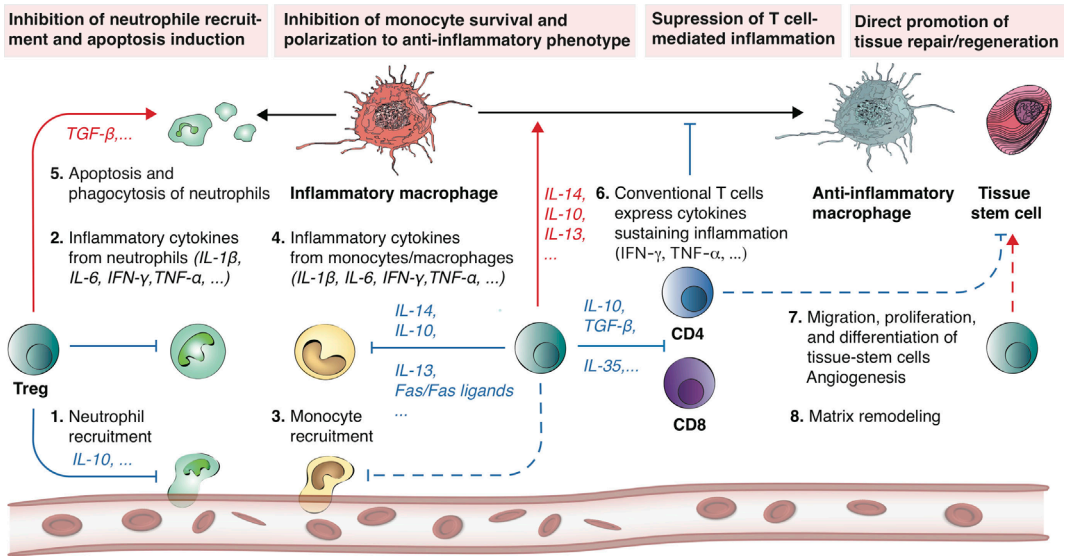

FIGURE 1 | Treg promote tissue repair and regeneration by modulating inflammation. Treg have demonstrated the ability to promote tissue repair and regeneration by controlling both the innate and adaptive immune systems. Following tissue injury, a cascade of immune events is triggered (steps 1–6) until a new tissue is formed (steps 7–8). Treg are involved in all these different steps. At the onset of inflammation, Treg can neutralize inflammatory cytokine secretion (e.g., IL-6, IFN-γ, TNF-α, and IL-1β) by inhibiting neutrophil extravasation *via* IL-10. In addition, Treg are able to promote apoptosis of neutrophils and encourage phagocytosis of dead neutrophils by macrophages. Concomitantly, Treg further inhibit monocyte activity, survival, and stimulate macrophage polarization toward an anti-inflammatory phenotype (M2) *via* the release of anti-inflammatory cytokines (e.g., IL-4, IL-10, IL-13). Similarly, Treg have the natural ability to suppress CD4 and CD8 T cell-mediated inflammation (*via* IL-10, TGF-β, and IL-35). Overall, these Treg-mediated mechanisms result in the inhibition of neutrophil, inflammatory macrophage, as well as CD4 and CD8 T-cell activity, which is generally favorable for tissue repair and regeneration. Dashed lines indicate a hypothetical mechanism. Red arrows indicate induction, while blue arrows indicate inhibition.

(32). These studies indicate that Treg-mediated modulation of neutrophil behavior and activity is an important step toward regulating tissue healing.

Asides from neutrophils, Treg interact with other key innate immune cells involved in the inflammatory response such as macrophages. In addition to being scavengers that phagocytose cellular debris including apoptotic neutrophils and other cells, macrophages have been shown to play a pivotal role in tissue repair and regeneration. As a remarkable example, salamanders are well-known to be able to regenerate limbs, but depletion of macrophages leads to failure of limb blastemal formation and regeneration (33). Similarly, genetic ablation of macrophages during blastemal proliferation leads to failure of tail fin regeneration in adult zebrafish (34). In mice, depletion of macrophages leads to excessive fibrosis and lack of neoangiogenesis, resulting in failure in neonatal heart regeneration after myocardial infarction (MI) or apex resection (11, 24). Likewise, macrophages are important for cardio protection driven by cardiosphere-derived cells (CDCs), a stem-like population derived from cardiac biopsies *ex vivo*. Indeed, systemic depletion of macrophages with clodronate abolishes CDC-mediated cardioprotection and inhibits their regenerative capability in adult hearts after MI (35).

Importantly, during tissue healing, there are at least two different subsets of monocyte-derived macrophages, namely M1 and M2 macrophages. M1 are pro-inflammatory macrophages usually induced by IFN-γ or TNF-α, while M2 are anti-inflammatory usually induced by IL-4/IL-13 or IL-10. In this context, Treg are important regulator of macrophage phenotypes and functions (36–38). For example, monocytes cocultured with Treg produce decreased level of TNF-α and IL-6 in response to LPS; and the inhibition is associated with secretion of IL-10, IL-4, and IL-13 by Treg (39). Additionally, coculture of monocytes with Treg induces macrophages to polarize toward a M2 phenotype characterized by the upregulation of CD206, CD163, and decreased expression of HLA-DR (40). Treg can attenuate tissue injury and help tissue repair also by modulating macrophage activity and survival. For example, in a chronic kidney disease model, Treg protect kidney injury through inhibition of macrophage activity, which is dependent on Treg-derived TGF-β (41). In this context, Treg also inhibit monocyte survival through the Fas/FasL pathway (41).

Treg FACILITATE TISSUE HEALING *VIA* REGULATION OF CONVENTIONAL T–CELL ACTIVITIES

Mounting evidence suggest that conventional T-cells are most likely detrimental for tissue healing (42). For example, CD4- and CD8-deficient mice have improved renal function in renal ischemia reperfusion model. SCID mice, which lack lymphocytes have significantly decreased intestinal leakage of albumin compared to wild-type mice after mesenteric artery ischemia and reperfusion (43, 44). In a MI model, $CD8^+$ cytotoxic T-cells can respond to cardiomyocytes after being exposed to autoantigen *in vivo* and kill healthy cardiomyocytes *in vitro* (45, 46).

Moreover, *Rag*$^{-/-}$ mice, which lack T-cells have significantly smaller infarct size compared to control mice (47). In the context of bone, conventional T-cells may inhibit regeneration by promoting osteoclast differentiation (48). In addition, recruitment of CD8$^+$ effector T-cells is correlated with delayed fracture healing and osteogenesis, due to secretion of IFN-γ and TNF-α (49). Deletion of CD8$^+$ T-cells in mouse osteotomy model enhances fracture healing while adoptive transfer of CD8$^+$ T-cells results in impaired healing (49).

The negative role of conventional T-cells in tissue injury is most likely mediated by the expression of inflammatory cytokines such as TNF-α and IFN-γ (50–52), but Treg can suppress conventional T-cells through various mechanisms including secretion of anti-inflammatory cytokines such as IL-10, TGF-β, and IL-35 (53–56). For example, it has been shown that deletion of Treg increase CD4$^+$ and CD8$^+$ cell number in the heart injury zone. In this context, both CD8$^+$ and CD4$^+$ T-cells show an increased secretion of IFN-γ and TNF-α, suggesting that Treg not only decrease the infiltration of conventional T-cells, but also attenuate their activity (22). Yet, the mechanisms by which Treg modulate tissue repair and regeneration are most likely tissue-dependent. In the next sections, we will discuss the role of Treg in the repair and regeneration of various tissues including skeletal and heart muscle, skin, lung, bone, and the central nervous system (CNS) (**Figure 2**).

THE ROLE OF Treg IN TISSUE-SPECIFIC REPAIR AND REGENERATION

Skeletal Muscle

It has been shown that Treg accumulate in the skeletal muscle of acutely injured mice or in mdx model of Duchenne muscular dystrophy (18, 57). Normal repair of skeletal muscle is found to require local expansion of Treg, since Treg ablation following treatment with diphtheria toxin in *Foxp3*^{DTR} mice or following treatment with the depleting anti-CD25mAb targeting CD4$^+$CD25^{hi} Treg increases muscle damage in dystrophic mice (18, 57). Similarly, treatments that enhance Treg activities including complexes of recombinant IL-2 with anti-IL-2 mAb prevented muscle damage in dystrophic mice (18, 57). Comparing the transcriptome of Treg isolated from regenerating muscle and lymphoid tissues including spleen and lymph nodes, Treg from muscle, but not naïve Treg from lymphoid organ express the growth factor Amphiregulin that directly acts on muscle satellite cells *in vitro* and improves muscle repair *in vivo* (18). Depletion of Treg also leads to increased muscle inflammation characterized by an increased IFN-γ response and activation of M1 macrophages (57). Moreover, it has been shown *in vitro* that coculture of induced Treg with muscle satellite cells enhances muscle satellite expansion and inhibits their myogenic differentiation (16). Nevertheless, direct evidence of Treg converting satellite cells into muscle has yet to be demonstrated.

To date, factors, which contribute to the accumulation of Treg in damaged tissues remain elusive. Nevertheless, IL-33 has been shown to facilitate recovery after CNS injury (58) and to drive accumulation of Treg in visceral adipose tissue of lean mice (59) and damaged muscle in young mice (60). IL-33 acts on the suppression of tumorigenicity 2 (ST2) receptor of Treg. Treg devoid of ST2 due to Treg cell-specific ablation of the *Il1rl1* gene show impaired recruitment to injured muscle, resulting in delayed muscle regeneration (60). Moreover, aged mice with more severely impaired muscle repair are found to have less IL-33-dependent accumulation of Treg after acute injury compared to young mice (60). Thus, IL-33 is important in mobilizing Treg in muscle and supplementation of IL-33 can reverse these effects and facilitate muscle regeneration in aged mice (16, 60).

Heart Muscle

Scarring of cardiac tissue after MI is likely the most deadly injury in humans (61). MI leads to a loss of large number of cardiomyocytes that are unable to regenerate, which ultimately progresses to cardiac failure. Cardiomyocyte death results in replacement by scar tissues and ventricular remodeling that further compromises heart function. Early cardiac wound healing is characterized by infiltration of both innate (62) and adaptive (63) immune cells into the myocardium. In patients with acute MI, increased systemic markers of inflammation correlate with higher mortality (64). Moreover, infiltration of activated CD4$^+$CD25$^+$ T-cells has been observed in the infarcted and remote regions of myocardium and heart-draining lymph nodes in patients with MI (63, 65). It has also been found that T-cells become activated in patients with coronary artery disease or a history of MI (65, 66). Nevertheless, the role of T-cells during pathogenesis or healing of the human heart is yet to be identified.

In mouse models of CD4$^+$ T-cell deficiency, including CD4 or MHC-II knockout mice, or TCR specific for an irrelevant ovalbumin-derived peptide in transgenic OTII mice, CD4$^+$ T cell-deficient mice show increased cardiac inflammation, impaired wound healing, left ventricular remodeling, and impaired survival (63). Although myocardial antigens are minimally accessible by the immune system, both *MhcII*$^{-/-}$ and OT-II mice have a higher rate of myocardial ruptures and mortality than wild-type mice. This suggest that CD4$^+$ T cells are activated after MI driven by recognition of cardiac autoantigens on MHC-II and facilitate healing of the myocardium in an antigen-specific manner (63).

Indeed, T-cells specific for myocardial proteins exist in mice. Both immunization with troponin or myosin-derived peptides, and adoptive transfer of myosin-derived peptides loaded dendritic cells induce myocarditis in susceptible mice (67, 68). CD4$^+$ T-cells are also found to be reactive to troponin, a complex comprised of three regulatory proteins troponin-C, -I, and -T, which are integral to cardiac muscle contractility (69). Moreover, the cardiomyocyte-specific protein, α-myosin heavy chain (α-MHC), is not expressed within the thymus of mice and human, and it resembles a non-self protein that activates CD4$^+$ T-cells after MI (70). Thus, tolerance to α-MHC reactive T-cells is probably maintained by Treg to prevent autoimmunity after MI. The detrimental role of conventional CD4$^+$ T-cells in MI healing most likely involves the adenosine receptor, since adenosine receptor depleted CD4$^+$ T-cells are not able to recapitulate the injurious action of CD4$^+$ T cells (47). *In vitro* activated Treg cells attenuate myocardial injury through expression of CD39, which promote

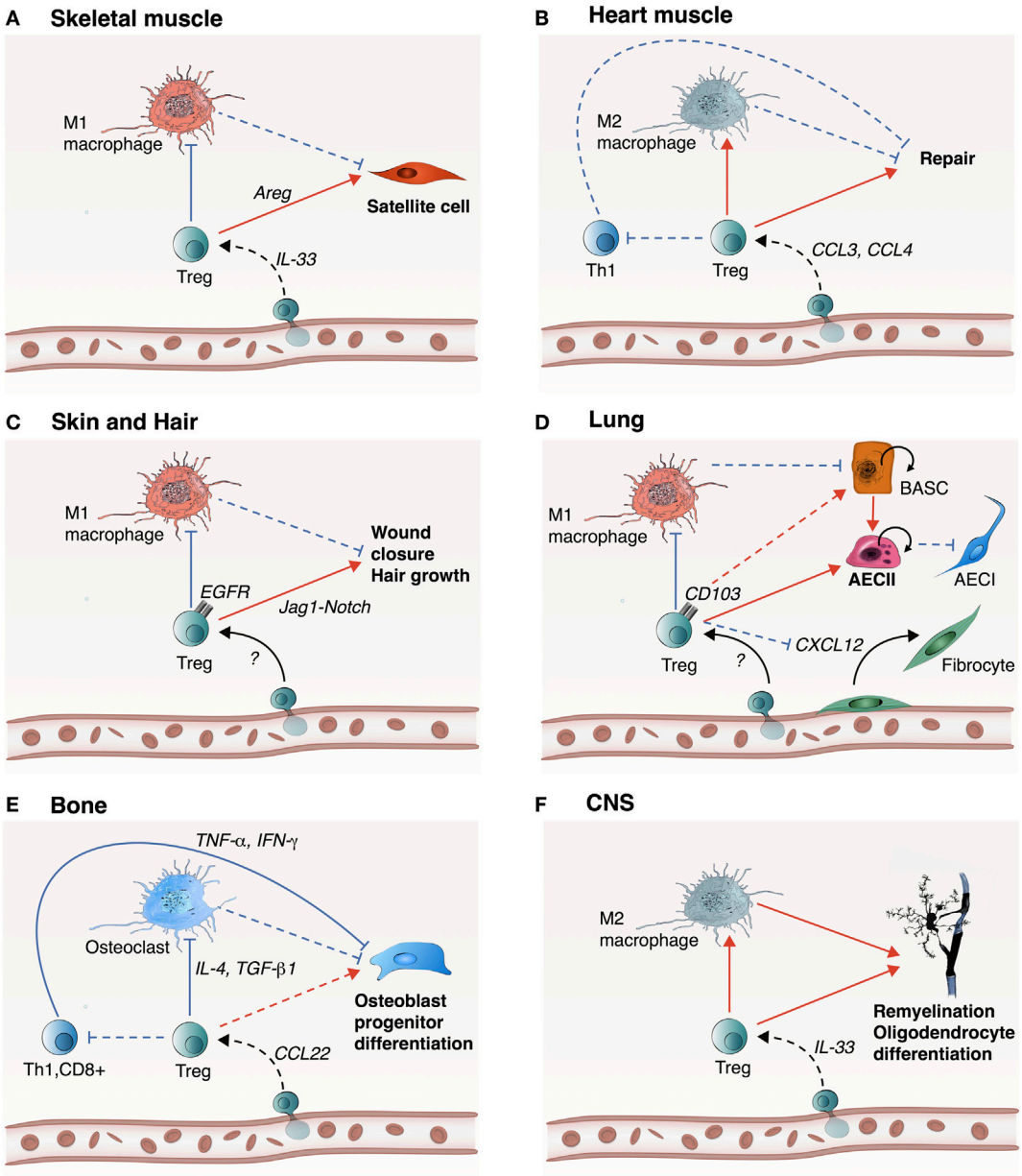

FIGURE 2 | Treg likely promote tissue repair and regeneration in a tissue-specific manner. Treg play an important role in the repair and regeneration of skeletal muscle, heart muscle, skin and hair, lung, bone, and central nervous system (CNS). **(A)** In skeletal muscle, IL-33 participates to Treg recruitment into the site of injury. Treg inhibit M1 macrophage-mediated inflammation, which promote transition to the resolution phase. Treg also directly activate satellite cell proliferation and differentiation through Areg. **(B)** In the heart, Treg are recruited via CCR5 signaling (e.g., CCL3 and CCL4) allowing inhibition of Th1 cell activity and inhibition of M1 macrophages. **(C)** In skin and hair, mechanism of Treg recruitment is still unknown, but upon recruitment, Treg inhibit M1 macrophage inflammatory activity and promote wound closure and hair growth *via* the Jag1-Notch signaling pathway. **(D)** In the lung, Treg inhibit M1 macrophage inflammatory activity and encourage proliferation and differentiation of damaged alveolar type 2 epithelial cells (AECII) into AECIs. This step can be mediated by Areg or CD103 to E-cadherin ligand-receptor binding. Alternatively, Treg could potentially activate progenitor bronchioalveolar stem cells (BASCs) to differentiate into AECII cells. Concurrently, Treg prevent fibrosis by inhibiting fibrocyte recruitment and proliferation *via* CXCL12. **(E)** In the bone, Treg are most likely recruited *via* CCL22, which act on inhibiting Th1, $CD8^+$, and M1 macrophages to support osteoblast progenitor differentiation. **(F)** In CNS, Treg are recruited by IL-33 and play a reparative role by encouraging M2 macrophage polarization to facilitate re-myelination and differentiation of oligodendrocytes. Treg may also directly act on oligodendrocytes *via* CCN3. Dashed lines indicate a hypothetical mechanism. Red arrows indicate induction, while blue arrows indicate inhibition.

extracellular degradation of nucleotides to form adenosine (71). Therefore, Treg in heart may function through CD39-mediated adenosine formation.

Treg also improve healing after MI by modulating monocytes and macrophages (22). Treg depletion in *Foxp3*^{DTR} mice or following treatment with anti-CD25 mAb show increased infiltration of

M1 macrophages, reduced cardiac function, and pronounced left ventricular dilation after MI (22). On the other hand, preferential induction of Treg *via* treatment with superagonistic anti-CD28 mAb leads to increased Treg infiltration into the infarcted myocardium after MI. The higher number of Treg promotes macrophages to polarize toward a M2 phenotype in the healing myocardium and reduce ventricular ruptures, which lead to better survival (22). Mechanistically, CCR5 is associated with Treg recruitment as CCR5 knockout mice show impaired Treg infiltration as well as adverse remodeling and cardiac deterioration after MI (72). Therefore, CCR5-mediated Treg recruitment restrains inflammation, excessive matrix degradation, and adverse remodeling after MI (72).

Skin and Hair

Layers of murine and human skin contain a large number of resident Treg (73–75). During a short-defined window of postnatal development, Treg migration to neonatal skin is important for the establishment of immune tolerance to commensal microbes (73). Upon entry to the skin through CCL20/CCR6 mediated migration, Treg localize and accumulate in hair follicles (76). Marked accumulation of $CD4^+$ T-cells are observed in wounded skin with peaked infiltration at day 7 following injury. Interestingly, the majority of $CD4^+$ T-cells are highly activated Treg characterized by increased expression of CD25, CTLA-4, and ICOS (77). Treg depletion following treatment with diphtheria toxin in *Foxp3*^{DTR} mice after skin wounding results in significant attenuation of wound closure accompanied by increased tissue granulation and overlying eschar, indicating that Treg facilitate skin wound healing (77). Treg depletion also leads to an increased number of IFN-γ producing T-cells with augmented accumulation of proinflammatory macrophages in wounded skin. Moreover, ablation of epidermal growth factor receptor (EGFR) signaling in Treg using the *Foxp3-Cre*;*EGFR*$^{fl/fl}$ mouse model results in reduced Treg infiltration in the wounded skin and significantly delayed wound closure, indicating that the EGFR pathway plays a role in Treg activation and function during skin wound healing (77).

In alopecia areata disease displaying phenotype of hair follicle regeneration, genome-wide association studies have revealed single nucleotide polymorphisms in genes including *Cd25*, *Ctla4*, *Eos*, and *Foxp3*, which are important in differentiation and function of Treg (78–80). Treg in skin preferentially localize to hair follicles (81, 82) and are more abundant in the resting telogen than growing anagen phase during hair follicle cycling (17). More importantly, in the telogen phase, Treg display a highly activated phenotype. Transient or constant ablation of Treg in *Foxp3*^{DTR} mice following treatment with diphtheria toxin leads to markedly reduced anagen induction in skin and subsequently reduced hair regrowth, indicating the important role of Treg in facilitating hair follicle regeneration by promoting the telogen-to-anagen transition (17). Immunofluorescence microscopy on dorsal skin derived from *Foxp3*^{GFP} reporter mice further revealed that Treg preferentially localize to hair follicular stem cell niche, promoting proliferation and differentiation of hair follicular stem cells (17). By comparing the transcriptome of Treg derived from telogen skin and skin-draining lymph nodes, Treg of the skin expressed more *Jag1* lymph node-Treg. Conditional ablation of *Jag1* in Treg of *Foxp3-Cre*$^{+/+}$;*Jag1*$^{fl/fl}$ mice significantly attenuates hair follicular stem cell proliferation, suggesting that the Jag1–Notch signaling pathway is essential in facilitating Treg-mediated hair follicle regeneration (17).

Lung

As microbes and other airborne materials can be frequently aspirated into the lung, pulmonary disease is easy to develop when the pulmonary immunity fails to protect the lungs during infections (83). However, the role of Treg during lung infections has been investigated in mouse models and has resulted in contradictory findings. It has been shown that patients with acute respiratory distress syndrome have increased Treg in their bronchoalveolar lavage fluid, suggesting that Treg play a role in the disease (21). In preclinical models of lung injury, T-cell deficient mice (*Rag-1*$^{-/-}$) showed delayed lung resolution governed by high lung permeability as well as elevated number of neutrophils and macrophages, indicating that T-cells may play a reparative role during lung injury resolution (21). Moreover, further analysis has shown that infiltration of $CD4^+CD25^+FOXP3^+$ Treg in the alveolar compartment increases upon LPS instillation (21). Furthermore, adoptive transfer of wild-type $CD4^+CD25^+$ splenocytes following intratracheal LPS instillation into *Rag-1*$^{-/-}$ mice successfully facilitates lung injury resolution, suggesting that Treg could serve as a rescue therapy after acute lung injury (21). Indeed, Treg play a central role in lung resolution, since adoptive transfer of Treg from wild-type into *Rag-1*$^{-/-}$ mice has shown to decrease the number of fibrocytes in LPS-treated lungs. Mechanistically, Treg reduce lung epithelial CXCL12 concentration which is responsible for $CXCR4^+$ fibrocyte recruitment (84). In addition, Treg mediate resolution by stifling pro-inflammatory macrophage response and ultimately promote bronchioalveolar stem cells (BASCs) proliferation (84).

The adult lung has a remarkable regenerative potential after injury (85, 86). The alveolar compartment comprises largely (90–95%) of alveolar type I cells (ATI) involved in gas exchange and to a lesser amount (7%) of type II cells (ATII), which are involved in immune regulation, repair, and recovery (87). These cell types derive from BASCs found at the bronchioalveolar duct junction (88). In a left unilateral pneumonectomy mouse model, surgical removal of the left lung induces mass expansion in the intact lobes of the remaining right lung (89). This extravagant alveologenesis process is shown to be dependent on lung epithelial proliferation, specifically through ATII cells responsible for maintaining ATI number through differentiation (89). In acute lung injury or partial pneumonectomy models, it has been shown that epithelial proliferation during lung recovery is significantly impaired after specific elimination of Treg in *Foxp3*^{DTR} mice following diphtheria toxin treatment (90). Lung epithelial proliferation is strongly correlated with Treg number after injury and Treg promote ATII proliferation through binding of their surface integrin CD103 to E-Cadherin expressed by epithelial cells (90). Furthermore, the growth factor amphiregulin (Areg) expression by Treg also seems to play a non-redundant role in lung repair (18). Rapid increase in expression level of Areg in lung tissues is observed at day 3 postviral infection. Using *Foxp3-Cre*;*Areg*$^{fl/fl}$

mice in which Areg is specifically ablated in Treg, it has been shown that the immunosuppressive function of Treg is preserved during antiviral immune responses. However, in the absence of Areg production by Treg, impaired recovery of lung function has been found in *Foxp3-Cre;Areg*$^{fl/fl}$ mice. Interestingly, by coculturing Treg with IL-18 or IL-33, it has been shown that Areg is induced by activation of IL-18R or ST2, instead of being activated through the TCR signaling pathway (91).

Bone

The adaptive immune system has been shown to play an important role in bone regeneration. Compared to most tissues, bone is capable of healing without scar tissue formation. The homeostasis of bone is mediated mainly by the interaction between osteoblasts which form bone and osteoclasts which resorb bone. Osteoblasts are principally differentiated from progenitors such as mesenchymal stem cells (MSC), while osteoclasts originate from bone marrow-derived monocytes. Interestingly, MSC can induce Treg from naïve T-cells and promote Treg proliferation through HO-1 (92). While CD3$^+$ T-cells support peripheral blood mononuclear cell differentiation into osteoclasts *in vitro*, Treg inhibit such a differentiation through paracrine signaling of TGF-β and IL-4 (93, 94). Moreover, the number of Treg in peripheral blood is inversely correlated to serum marker of osteoclastogenesis in normal human and rheumatoid arthritis patients. *In vivo* experiments have also shown that Treg protect TNF-α-induced bone destruction and ovariectomy-induced bone loss (95, 96). Treatment with superagonistic anti-CD28 mAb ameliorates TNF-α-induced arthritis and increases bone mass in wild-type mice. The protective role of Treg in bone loss is most likely the result of impaired osteoclast differentiation and bone resorption *via* inflammatory cytokines. For example, in an actinobacillus actinomycetemcomitans-induced canine model, Treg are recruited to the site of injury by CCL22 and decrease bone resorption through reducing inflammation (97). Interestingly, Treg may also directly promote osteoblast differentiation from progenitor cells. For instance, it has been demonstrated that Treg facilitate MSC-based bone regeneration by inhibiting CD4$^+$ conventional T-cells, which secrete IFN-γ and TNF-α (52, 98).

Central Nervous System

In mice deficient for CD4$^+$ or CD8$^+$ T-cells, remyelination is inhibited after lysolecithin injection, suggesting that CD4$^+$ and CD8$^+$ T-cells are required in remyelination of the CNS (99). In a myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis mouse model, Treg are found to expand in peripheral lymphoid compartment and accumulate in CNS (100). Even though infiltrating Treg fail to control autoimmune inflammation, it has been demonstrated that they promote myelin regeneration (25). Moreover, Treg-deficient mice show impaired remyelinataion and oligodendrocyte differentiation that can be rescued by adoptive transfer of Treg. IL-33 has been found to promote Treg recruitment into injured tissues, facilitating recovery after CNS injury. In addition, mice lacking IL-33 have impaired recovery after CNS injury, which is associated with reduced myeloid cell infiltrates and decreased induction of M2-assiociated genes at the injury site (58). Treg also promote oligodendrocyte progenitor cell differentiation and myelination *in vitro* and *ex vivo*. Interestingly, through proteome profiling of Treg conditioned media, nephroblastoma overexpressed, also known as CCN3, has been found to mediate Treg-driven oligodendrocyte progenitor cell differentiation and CNS myelination (25).

FUTURE PERSPECTIVES

Recently, exploring the function of the immune system during tissue repair and regeneration has gained a lot of interest in regenerative medicine. Nevertheless, we still have sparse knowledge on how immunity—in particular the adaptive immune system—controls the tissue healing process. For instances, what are the neo antigens, if any, released to initiate adaptive immunity-mediated tissue healing? Similarly, is adaptive immunity-mediated tissue healing an antigen-specific process? If so, how are T-cells recruited, activated and function in response to self-antigens during injury that are different from responding to non-self antigens? Currently, the development of treatments targeting the immune system is hindered by the lack of markers that specifically define distinct subsets of immune cells. Recent advances in single-cell genomics could offer unprecedented delineation of lineage-specific markers and function of various subsets of immune cells operating during tissue repair and regeneration.

Furthermore, it is still unclear why scars are absent in some tissues such as in bone, but are forming in others such as in heart. Understanding how both innate and adaptive immune cells interact with tissue-resident progenitor cells and myofibroblasts would shed light on developing therapeutic strategies for improving healing and regeneration in the clinic. Moreover, accumulating evidence has shown that the function of the immune system declines with age (101, 102). Given that the immune system plays a crucial role in tissue repair and regeneration, whether the reduced tissue repair capacity is related to a degenerated immune system during aging awaits further investigations.

Overall, studies investigating the role of Treg during tissue regeneration have been largely based on the use of *Foxp3*^{DTR} mouse model (17, 18, 22, 25, 57, 77, 90). However, one caveat of using such model is that the mice develop spontaneous systemic autoimmunity when Treg are depleted for long term (103). Careful data analysis should also include gain-of-function experiments such as adoptive transfer of purified Treg into *Rag1*$^{-/-}$ mice to determine the role of Treg in tissue repair and regeneration.

From a regenerative point of view, one could control tissue Treg to promote regeneration. Treg of adipose tissues, skeletal muscle, and colonic lamina propria are the best characterized tissue Treg that maintain organismal homeostasis (104). Similar to regenerative Treg as aforementioned, IL-33 has been reported to expand tissue Treg in colonic lamina propria (105) that are well equipped to participate in local repair responses with expression of the tissue repair factor, amphiregulin. The exact role of these tissue Treg in intestinal regeneration awaits further investigations. Nevertheless, manipulating Treg for alleviating inflammatory diseases has been tested in several clinical trials. For instances, the use of low dosage of IL-2 in selective expansion of Treg in human patients (106) as well as *ex vivo* expansion and adoptive transfer

of Treg to treat Type 1 diabetes have been reported and tested in the clinic (107–109). Further studies are needed to investigate if these Treg-mediated strategies can also be utilized for inducing tissue regeneration.

Although it has been reported that superagonistic anti-CD28 mAb increases Treg infiltration or activities in mice, the use of humanized superagonisticanti-CD28 antibody TGF1412 caused cytokine storm, leading to organ failure in a previous trial (110). Furthermore, even though IL-33 plays an important role in recruitment and function of Treg in mice (60), IL-33 is dispensable in humans as individuals lacking IL-33 have no obvious health problems such as autoimmunity, indicating that the pro-regenerative function of IL-33 on Treg could also be different between mice and humans (111). Therefore, novel strategies in empowering Treg-mediated tissue regeneration for potential clinical uses would be needed in the future.

AUTHOR CONTRIBUTIONS

JL and KL wrote the manuscript; JT and MM made the figures; MM and KL revised the manuscript.

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Activation of the Regulatory T-Cell/Indoleamine 2,3-Dioxygenase Axis Reduces Vascular Inflammation and Atherosclerosis in Hyperlipidemic Mice

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T-cell activation is characteristic during the development of atherosclerosis. While overall T-cell responses have been implicated in disease acceleration, regulatory T cells (Tregs) exhibit atheroprotective effects. The expression of the enzyme indoleamine 2,3-dioxygenase-1 (IDO1), which catalyzes the degradation of tryptophan (Trp) along the kynurenine pathway, has been implicated in the induction and expansion of Treg populations. Hence, Tregs can reciprocally promote IDO1 expression in dendritic cells (DCs) *via* reverse signaling mechanisms during antigen presentation. In this study, we hypothesize that triggering the "Treg/IDO axis" in the artery wall is atheroprotective. We show that apolipoprotein B100-pulsed tumor growth factor beta 2-treated tolerogenic DCs promote *de novo* FoxP3$^+$ Treg expansion *in vivo*. This local increase in Treg numbers is associated with increased vascular IDO1 expression and a robust reduction in the atherosclerotic burden. Using human primary cell cultures, we show for the first time that IDO1 expression and activity can be regulated by cytotoxic T-lymphocyte associated protein-4, which is a constitutive molecule expressed and secreted by Tregs, in smooth muscle cells, endothelial cells, and macrophages. Altogether, our data suggest that Tregs and IDO1-mediated Trp metabolism can mutually regulate one another in the vessel wall to promote vascular tolerance mechanisms that limit inflammation and atherosclerosis.

Keywords: atherosclerosis, T-cell, regulatory T cell, IDO, tryptophan, kynurenine

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease initiated by the retention and accumulation of low-density lipoprotein (LDL) in the artery wall. Trapped in the intima, the lipid moiety of LDL or apolipoprotein B100 (ApoB100) have been identified as major drivers of this disease, triggering the response of vascular, innate, and adaptive immune cells (1). While overall T-cell responses have been implicated in the aggravation of atherosclerosis, regulatory T cells (Tregs)

have been identified as critical for protection against disease (2). Tregs can exert their antiatherogenic effects *via* the local secretion of anti-inflammatory cytokines, such as transforming growth factor beta (TGFβ) and interleukin 10 (IL-10), or by cell-to-cell contact (3).

We have previously shown that the immunomodulation of T-cell responses can reduce vascular inflammation and plaque formation (4–6). Indoleamine 2,3-dioxygenase-1 (IDO1), which is the rate-limiting enzyme catalyzing the production of metabolites in the Kynurenine pathway of tryptophan (Trp) degradation, has been implicated in the regulation of T-cell effector responses and the expansion of Tregs (7–9). The inhibition of Trp metabolism using the IDO inhibitor 1-methyl tryptophan (1-MT) or the genetic ablation of IDO1 in hypercholesterolemic mice results in a substantial increase in vascular inflammation and accelerated plaque formation (10, 11).

Pro-inflammatory signaling pathways, including toll-like receptors, tumor necrosis factor superfamily members, type I and II interferons, and the aryl hydrocarbon receptor, have been implicated in the regulation of IDO expression (12). Nevertheless, Treg signals, such as TGFβ and cytotoxic T-lymphocyte associated protein-4 (CTLA4), have been also shown to influence IDO1 expression in antigen-presenting cells (APCs) in animals and humans (13, 14).

In this study, we hypothesized that the activation of the "Treg/IDO axis" in the vascular wall can modulate atherosclerosis. We show that promoting the expansion of antigen-specific FoxP3$^+$ Tregs in the artery wall with an injection of tumor growth factor beta 2 (TGFβ$_2$)-treated and ApoB100-pulsed tolerogenic dendritic cells (DCs) leads to increased IDO1 expression and atheroprotection. Indeed, we show that CTLA4 is a major regulator of IDO1 expression and activity in vascular cells and macrophages. Our data reveal novel mechanisms underlying the maintenance of immunohomeostasis in the vascular wall. Thus, the induction of the "Treg/IDO axis" emerges as a promising therapeutic approach for the prevention and treatment of atherosclerotic cardiovascular diseases (CVDs).

MATERIALS AND METHODS

Animals

Human ApoB100-transgenic *Ldlr*$^{-/-}$ mice [*HuBL*, *B6.C57BL/6XSJL-*$^{Tg(huB100tm)}$*0.129S7-Ldlr*^{tm1Her} (15, 16)] were used for the generation of the bone marrow-derived DCs and the atherosclerosis experiments. T cells from C57BL6/J mice were used in the Treg conversion assays.

Preparation of ApoB100

Low-density lipoprotein (1.019−1.063 g/mL) was isolated from pooled plasma from healthy donors by sequential ultracentrifugation as previously described (17). ApoB100 was isolated by the addition of four parts of methanol, one part of chloroform, and three parts of water to one part of LDL. Then, the mixture was vortexed and centrifuged at 9,000 × *g* for 10 min, which resulted in protein precipitation at the chloroform–methanol–water interphase. Then, ApoB100 was dissolved in sodium dodecyl sulfate, filtered using a PD-10 column (GE Healthcare Life Sciences, Uppsala, Sweden), and purified by high-pressure liquid chromatography using a Superdex200 (GE Healthcare Life Sciences, Uppsala, Sweden) size-exclusion column (0.5 mL/min in Tris-buffered saline, pH 7.6).

Preparation of Bone Marrow-Derived DCs

The DCs were isolated as previously described (5). Briefly, bone marrow cells from the femur and tibia bones of *HuBL* mouse donors were depleted of red blood cells and cultured at 37°C and 7.5% CO_2 for 8 days in medium (DMEM, 10% FCS, 50 U/mL penicillin, 50 g/mL streptomycin, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine) supplemented with 10 ng/mL IL-4 and 10 ng/mL GM-CSF (PeproTech, NJ, USA). The generated DCs were purified by positive selection using CD11c magnetic cell-sorting kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

De Novo Induction of Tregs *In Vitro*

The conversion of Tregs *in vitro* was investigated using CD11c$^+$ DCs that were incubated with either 5 ng/mL TGFβ$_2$ (R&D Systems, MN, USA) or 10 μg/mL IL-10 (R&D Systems, MN, USA) for 24 h. A group without cytokine treatment was used as a control. After washing, the DCs were cocultured at 37°C and 7.5% CO_2 for 48 h with CD4$^+$CD25$^-$ naïve T cells obtained by negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany) from spleens from C57BL6/J mice. The polyclonal conversion of Tregs was induced by stimulation with 1 μg/mL anti-CD3 (R&D Systems, MN, USA) and 2 μg/mL anti-CD28 (R&D Systems, MN, USA). After 48 h, the percentage of CD4$^+$CD25$^+$FoxP3$^+$Ki67$^+$ cells was assessed by flow cytometry.

Treg Generation *In Vivo* by TGFβ$_2$ Tolerogenic DCs

We have previously shown that IL-10-generated tolerogenic DCs can induce antigen-specific Treg formation *in vitro* and *in vivo* (5). In this study, we show that TGFβ$_2$ has a superior capacity to induce Treg conversion *in vitro*; thus, these DCs were selected for the *in vivo* experiments. The DCs, which were generated as previously described, were incubated in tissue culture dishes with 5 ng/mL TGFβ$_2$ (R&D Systems, Minneapolis, MN, USA) with or without 25 μg/mL ApoB100, in serum-free DMEM medium containing insulin, human transferrin, selenous acid (1:100 ITS Premix, Biosciences, Franklin Lakes, NJ, USA), 1 mmol/L sodium pyruvate (Gibco Invitrogen, Carlsbad, CA, USA), 1 mg/mL bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), 1 mmol/L non-essential amino acids (Sigma-Aldrich, Stockholm, Sweden), 10 mmol/L HEPES (Gibco Thermo Fisher Scientific, MA, USA), and 50 g/mL gentamicin sulfate (Sigma-Aldrich, St. Louis, MO, USA) at 37°C and 5% CO_2. After 4 h, 0.1 ng/mL lipopolysaccharide was added, and the cells were incubated for an additional 14 h. Finally, the DCs were washed with DMEM, maintained on ice, and injected into recipient mice within 1 h. Cytokine secretion, including IL-10, IL-12, and TNF, by the DCs was analyzed in the supernatants of cultures by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Eleven-week-old male HuBL mice were injected with 2.5×10^5 DCs that had been loaded or not with ApoB100 and treated with or without TGFβ_2. Five days after the DC transfer, the mice were fed a Western diet (corn starch, cocoa butter, casein, glucose, sucrose, cellulose flour, minerals, and vitamins; 17% protein, 21% fat, 0.15% cholesterol, 43% carbohydrates, 10% H_2O, and 3.9% cellulose fibers; R638 Lantmännen, Kimstad, Sweden) for 10 weeks.

Tissue Processing, Immunostaining, and Lesion Analysis

After sacrifice, blood was collected by cardiac puncture, and vascular perfusion was performed using sterile RNase-free PBS. The abdominal aorta was dissected and snap-frozen for the subsequent RNA isolation. The heart and aortic arch were dissected and preserved for the immunohistochemistry and plaque analyses as previously described (18). *En face* lipid accumulation was determined in the thoracic aorta from the immunized mice using Sudan IV staining. The plaque area was calculated as the percentage of the total surface area of the thoracic aorta. The plaque cell markers in sections of aortic roots was evaluated using primary antibodies against vascular cell adhesion molecule 1 (VCAM-1) (all BD Biosciences, Franklin Lakes, NJ, USA); CD68 (AbD Serotec, Kidlington, UK), α-smooth muscle-actin (αSMA) (Abcam, Cambridge, UK); CD31 (Abcam, Cambridge, UK); FoxP3 (eBioscience, Thermo Fisher Scientific, MA, USA); and IDO1 (BioLegend, San Diego, CA, USA) that were applied to acetone-fixed cryosections, and against L-kynurenine (ImmuSmol, Pessac, France) that was applied to paraformaldehyde-fixed cryosections. The detection was performed using an ABC alkaline phosphatase kit (Vector Laboratories, Burlingame, CA, USA) or Envision system (Dako, Copenhagen, Denmark) as previously described (18, 19). Immunofluorescence staining was performed using goat anti-rat IgG (Dylight® 594) and horse anti-rabbit (Dylight® 488) as the secondary antibodies (Vector Laboratories, Burlingame, CA, USA), and nuclei were stained with DAPI (Sigma-Aldrich, MO, USA).

Plasma Analysis

The plasma cholesterol and triglyceride levels were measured using enzymatic colorimetric assays (Randox Laboratories, Crumlin, UK) according to the manufacturer's protocol.

Flow Cytometry Analysis

The characterization of the DC and T-cell phenotypes was performed by flow cytometry (CyAnTM; Dako, Glostrup, Denmark). Primary antibodies against murine CD11c, I-Ab, CD11b, CD205, CD86, Ki67 (all from BD Biosciences, NJ, USA) and FoxP3 (eBioscience, Thermo Fisher Scientific, MA, USA) were used. The results were acquired using FlowJo software (TreeStar software, Ashland, OR, USA).

Quantitative PCR

RNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany), reverse-transcribed, and amplified by real time-PCR using Assay-On-Demand primers and probes (Applied Biosystems, CA, USA). Hypoxanthine guanidine ribonucleosyl transferase was used as a housekeeping gene. The relative expression was calculated using the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ (sample) $- \Delta Ct$ (calibrator = average Ct of controls), and ΔCt is the average Ct of the housekeeping gene subtracted from the target gene Ct.

Human Macrophage Cultures

Peripheral blood was obtained from healthy volunteers at the Blood Central of Karolinska University Hospital, Stockholm, Sweden. Peripheral blood mononuclear cells were isolated using Lymphoprep™ gradient medium (density 1.077 g/ml; Axis-Shield, Oslo, Norway) according to the manufacturer's instructions. After a 1 h adherence step, the floating cells were discarded. The adherent monocytes were used to generate "M0" macrophages as previously described (20). Briefly, the monocytes were cultured for 6 days in medium [RPMI 1640, 50 U/mL penicillin, 50 g/mL streptomycin (Gibco Invitrogen, Carlsbad, CA, USA), and 10% fetal bovine serum] supplemented with 20 ng/mL M-CSF (R&D Systems, MN, USA). After 24 h of pre-stimulation with human recombinant IFNγ (400 U/mL), the cells were washed three times with PBS and incubated with medium alone, recombinant human CTLA4-Ig (hum/hum) or recombinant human IgG1-Fc isotype control (both from BioXCell, Lebanon, NH, USA) for 24 h. Unstimulated macrophages were used as controls. The supernatants were collected for the determination of IDO activity by HPLC.

Human Smooth Muscle Cell (SMC) Cultures

Commercial human aortic SMCs (Cascade Biologics, Life Technologies, CA, USA) were maintained in SMC medium (Lonza, Basel, Switzerland). After 24 h of pre-stimulation with human recombinant IFNγ (400 U/mL), the cells were washed three times with PBS and incubated with medium alone, recombinant human CTLA4-Ig (hum/hum) or recombinant human IgG1-Fc isotype control (both from BioXCell, Lebanon, NH, USA) for 24 h. Unstimulated SMCs were used as controls. The supernatants were collected for the determination of IDO activity by HPLC.

Human Umbilical Vein Endothelial Cell (HUVEC) Cultures

Human umbilical vein endothelial cells were cultured in Medium 199 supplemented with 20% (v/v) fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine, 1 μg/mL heparin (Sigma-Aldrich, St. Louis, MO, USA), and 10 μg/mL endothelial cell growth factor supplement (Sigma-Aldrich, St. Louis, MO, USA). Cells from passage 1 or 4 were used in all experiments. Cells grown to confluence were pretreated with INF-γ (400 U/mL) at 37°C for 24 h. After three washes with PBS, the cells were incubated with medium alone, recombinant human CTLA4-Ig (hum/hum) or recombinant human IgG1-Fc isotype control (both from BioXCell, Lebanon, NH, USA) for an additional 24 h. Unstimulated HUVECs were used as controls. The supernatants were collected for the determination of IDO activity by HPLC.

IDO Activity Assay

The Kyn to Trp ratio (Kyn/Trp) was used as a surrogate marker of IDO activity. The Trp and Kyn levels in the plasma and tissues were analyzed by isocratic liquid chromatography with ultraviolet detection as previously described (21).

Arterial IDO Quantification by Western Blotting

The total protein was extracted from frozen aortic root sections as previously described (22). Ten micrograms of extract were separated by SDS–PAGE (4–15%, Bio-Rad Laboratories, CA, USA) and transferred to PVDF membranes (GE Healthcare, Uppsala, Sweden). The membranes were probed for murine IDO using an M-48 antibody (BioLegend, San Diego, CA, USA). An α-tubulin quantification (anti-α-tubulin; Abcam, Cambridge, UK) was performed as a loading control.

Statistics

The non-parametric Mann–Whitney *U*-test was used for comparisons between two groups. Comparisons among more than two groups were performed using non-parametric Kruskal–Wallis ANOVA, followed by a Dunn's multiple comparison *post hoc* test. The correlations were calculated using the Spearman's rank test. The differences were considered significant at *P*-values < 0.05 (two-tailed). All statistical analyses were performed using GraphPad Prism version 6.0f for Mac OS X (GraphPad Software, Inc., CA, USA).

RESULTS

TGFβ$_2$ Induces a Potent Tolerogenic Phenotype in DCs

Bone marrow-derived DCs from HuBL mice were incubated in the presence or absence of TGFβ$_2$ and pulsed with or without ApoB100 as described in the methods. The untreated DCs were characterized as CD11c$^+$MHC-II^{high}CD11b$^+$DEC205$^+$ (the detailed phenotype of DCs is shown in Figure S1 in Supplementary Material). Upon TGFβ$_2$ treatment, the DCs exhibited a decreased surface expression of the co-stimulatory molecule CD86 and a modest reduction in the I-A^b MHC-II levels, compared with the controls (**Figure 1A**). Based on an analysis of the supernatants from these cells, we show that TGFβ$_2$-induced tolerogenic DCs secrete lower levels of IL-12 and produce higher levels of IL-10 independently of being loaded with or without ApoB100 (**Figures 1B–C**). A trend toward reduced TNF secretion was observed in the TGFβ$_2$-induced tolerogenic DCs (**Figure 1D**).

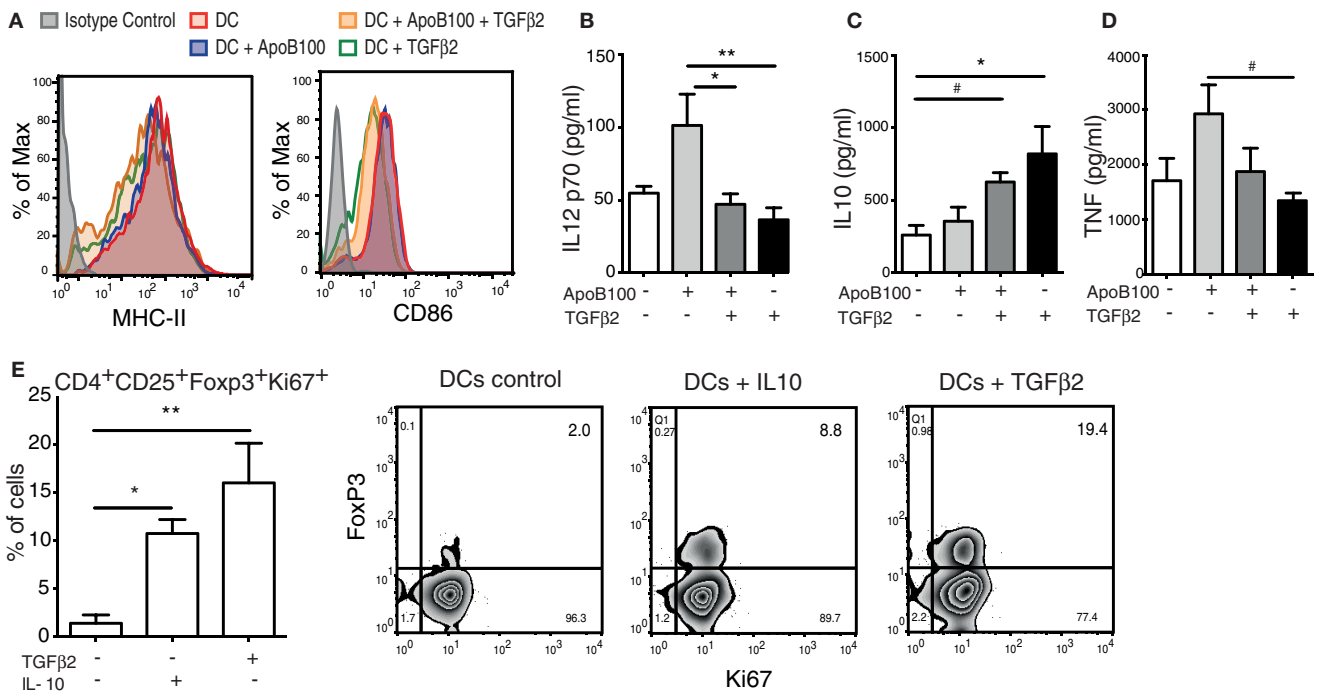

FIGURE 1 | Tumor growth factor beta 2 (TGFβ$_2$)-induced tolerogenic dendritic cells (DCs) generate *de novo* regulatory T cells (Tregs) *in vitro*. **(A)** Representative flow cytometry overlaid histograms of MHC-II and CD86 expression in bone marrow-derived DCs incubated with different treatments. **(B–D)** Levels of IL-12 p70, interleukin 10 (IL-10), and TNF from the supernatants of bone marrow DCs incubated with different treatments. The results are pooled data from four independent experiments (duplicate wells). Pooled cells from five mice were used in each experiment. Unstimulated DCs were used as a control. **(E)** Proliferation and *de novo* induction of Tregs (CD4$^+$CD25$^+$FOXP3$^+$KI67$^+$ cells) obtained from naïve CD4$^+$ T cells co-incubated with DCs pretreated with TGFβ$_2$, IL-10, or DCs alone. The results are pooled data from four independent experiments (duplicate wells). Unstimulated DCs were used as a control. Right panels: representative plots of cytometric analysis of proliferating *de novo* induced Tregs after coculture with DCs treated with different cytokines. The data are expressed as the mean ± SEM ($^{\#}P = 0.05$, $^*P < 0.05$, and $^{**}P < 0.01$).

Notably, TGFβ$_2$-induced tolerogenic DCs presented also increased IDO1 protein expression (Figure S2 in Supplementary Material).

Next, we evaluated the capacity of the TGFβ$_2$-induced tolerogenic DCs to induce the *de novo* expression of Foxp3 in naïve CD4$^+$CD25$^-$ T cells *in vitro*. Compared with the untreated or IL-10-treated DCs, the TGFβ$_2$-treated DCs showed a superior capacity to induce Tregs (**Figure 1E**). Thus, TGFβ$_2$-induced tolerogenic DCs were selected to be used as a tool to expand ApoB100-specific Tregs *in vivo*.

Injection of TGFβ$_2$-Treated ApoB100-Pulsed DCs Increases Treg Numbers in Atherosclerotic Plaques

HuBL mice were divided in four groups that received a single intravenous injection of (i) untreated, (ii) ApoB100-pulsed, (iii) TGFβ$_2$-treated and ApoB100-pulsed, or (iv) TGFβ$_2$-treated DCs. The immunohistochemistry analysis of the lesions revealed that only the TGFβ$_2$-treated ApoB100-pulsed DCs substantially increased the FoxP3$^+$ Treg numbers in the plaques (**Figure 2A**). Consistently, we observed an increased expression of Treg markers, including the mRNA levels of Foxp3, the anti-inflammatory cytokine IL-10, and the co-inhibitory molecule CTLA4, in para-aortic lymph nodes from the same group (**Figure 2B–D**).

Treg Expansion Is Associated With Increased Arterial IDO1 Expression and Activity

Because increased antigen-specific Treg infiltration was observed in the vascular wall, we investigated whether IDO1 expression was concomitantly affected. Indeed, the Treg/IDO axis was triggered in the mice injected with TGFβ$_2$-treated ApoB100-pulsed DCs. These mice exhibited increased arterial expression of IDO1 at the mRNA (**Figure 3A**) and protein levels (**Figure 3B**). In line with these data, mice receiving TGFβ$_2$-treated ApoB100-pulsed DCs presented significantly increased L-kynurenine staining in their plaques (**Figure 3C**), suggesting a local increase in Trp metabolism in the vascular wall. A similar trend of increased IDO1 expression was observed in the para-aortic lymph nodes (Figure S3 in Supplementary Material).

Subsequently, we investigated IDO1 cell localization in the arteries in the same group. IDO1 co-localized with SMC (αSMA), endothelial cell (CD31), and macrophage (CD68) markers (**Figure 3D**). Whether the different DC-treatments systemically influenced IDO1 expression was first evaluated in mice spleens, but no differences were observed (Figure S3 in Supplementary Material). Consistently, no differences were observed in the plasma Kyn/Trp ratio among the groups (Figure S4 in Supplementary Material).

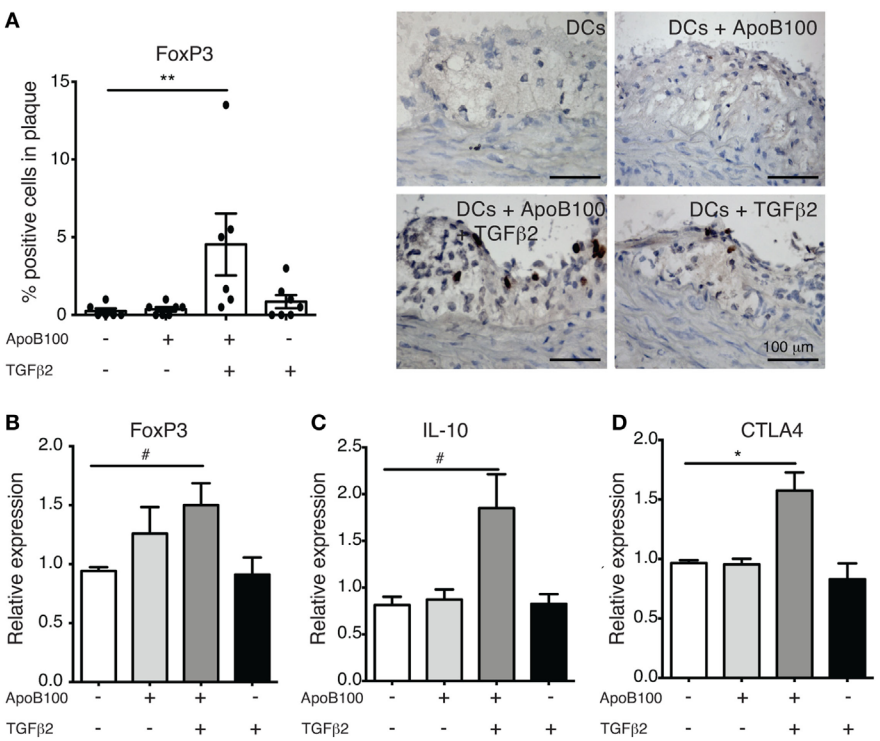

FIGURE 2 | Tumor growth factor beta 2 (TGFβ$_2$)-apolipoprotein B-100 (ApoB100)-loaded dendritic cells (DCs) increase regulatory T cell numbers in atherosclerotic plaques. **(A)** Analysis of immunostaining for FoxP3-positive cells in atherosclerotic plaques from mice injected with DCs alone ($n = 6$), DCs loaded with ApoB100 ($n = 7$), DCs loaded with ApoB100 and treated with TGFβ$_2$ ($n = 6$), and DCs treated with TGFβ$_2$ alone ($n = 7$). Right panels: representative microphotographs of staining of FOXP3-positive cells. **(B–D)** mRNA expression of FoxP3, interleukin 10 (IL-10), and cytotoxic T-lymphocyte associated protein-4 (CTLA4) in para-aortic lymph nodes from mice treated with different stimuli. The data are expressed as the mean ± SEM ($^{\#}P = 0.05$, $^{*}P < 0.05$, and $^{**}P < 0.01$).

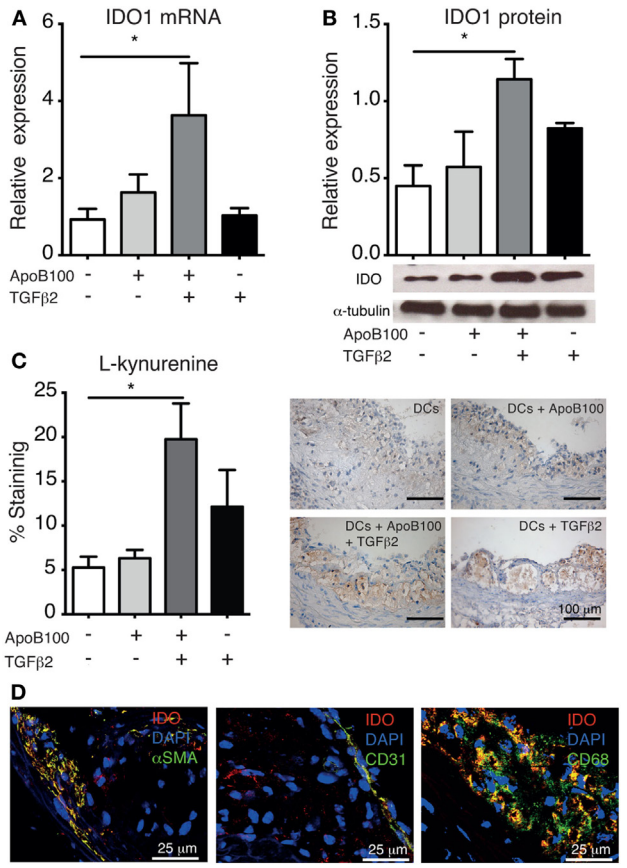

FIGURE 3 | Tumor growth factor beta 2 ($TGF\beta_2$)-apolipoprotein B-100 (ApoB100)-loaded dendritic cells (DCs) increase arterial indoleamine 2,3-dioxygenase-1 (IDO1) expression. **(A)** IDO mRNA expression in abdominal aortas from mice treated with DCs alone ($n = 6$), DCs loaded with ApoB100 ($n = 7$), DCs loaded with ApoB100 and treated with $TGF\beta_2$ ($n = 6$), and DCs treated with $TGF\beta_2$ alone ($n = 7$). **(B)** Analysis of IDO1 protein expression in aortic roots. Right panel: representative immunoblot of IDO1 and alpha tubulin in aortic roots from mice treated with the different DCs. **(C)** Analysis of L-kynurenine-positive immunostaining in atherosclerotic plaques from different DC-treated mice. Right panels: representative microphotographs of L-kynurenine staining in atherosclerotic plaques from aortic roots. The data are expressed as the mean ± SEM (*$P < 0.05$). **(D)** Representative microphotographs of IDO co-localization (red) with SMA-positive smooth muscle cells (green, left panel), CD31-positive endothelial cells (green, middle panel), and CD68-positive macrophages (green, right panel).

Activation of the Treg/IDO Axis Reduces Atherosclerosis and Vascular Inflammation

We evaluated whether the Treg/IDO axis could influence plaque development in thoracic aortas from the four DC-treated groups. The mice receiving the $TGF\beta_2$-treated ApoB100-pulsed DCs exhibited a 75% decrease in the surface lesion area in the thoracic aorta compared with that in the other groups (**Figure 4A**). The reduction in the lesion area was associated with a significant reduction in plaque CD68 macrophage infiltration and VCAM-1 expression (**Figures 4B–C**). No differences in aortic M1 or M2 markers (Figure S5 in Supplementary Material), body weight and total plasma lipid levels were observed among the groups (**Table 1**).

CTLA4-Ig Enhances IDO1 Expression and Activity in Activated Human Cultured Macrophages, SMCs, and Endothelial Cells

Cytotoxic T-lymphocyte associated protein-4 has been identified as a key effector molecule of Tregs (23). CTLA4 binding to CD80/CD86 has been shown to regulate cytokine-dependent IDO1 expression in DCs, leading to the mutual regulation of them and the T cells expressing CTLA4 (14). However, whether similar mechanisms operate in cells other than DCs have never been explored.

Expectedly, the IFNγ priming of human macrophages, SMCs, and endothelial cells upregulated IDO1 expression and activity *in vitro* (**Figure 5**; Figure S6 in Supplementary Material). A distinct fold change in IDO1 mRNA expression was observed among the different cell lines upon IFNγ stimulation (>1,000-fold in the SMCs; >30-fold in the macrophages; and 2-fold in the HUVECs). Interestingly, the concomitant treatment of these cells with CTLA4-Ig further increased the IDO mRNA levels (**Figures 5A–C**). Moreover, a significant increase in IDO activity was observed in the CTLA4-Ig-stimulated cells (**Figures 5D–F**), particularly in the SMCs. Notably, pre-stimulation with IFNγ led to increased CD80 mRNA levels on SMCs and HUVECs (Figure S7 in Supplementary Material), and no effects of CTLA4-Ig were observed in the absence of IFNγ simulation (**Figure 5**). CD86 mRNA was not influenced by treatments in macrophages and was undetectable in SMCs and HUVECs (Figure S7 in Supplementary Material).

DISCUSSION

In this study, we employed tolerogenic DC-based immunotherapy to induce Tregs specific to the atherosclerosis-related antigen ApoB100. This treatment led to an increased number of $FoxP3^+$ Tregs and increased IDO1 expression in the vessel wall. Hence, the concomitant induction of Tregs and IDO1 was followed by a reduction in the atherosclerotic burden.

Tolerogenic DC-based immunotherapy could be a promising strategy to modulate atherosclerosis (5, 24). For example, we showed that IL-10 renders DCs a tolerogenic phenotype and the capacity to induce antigen-specific Tregs *in vivo* and prevent disease (5). While this approach remains at the experimental level in the cardiovascular field, tolerogenic DC-based clinical trials for the treatment of autoimmune diseases, including type 1 diabetes, rheumatoid arthritis (RA), multiple sclerosis, and Crohn's disease, are underway (25).

In this study, tolerogenic DCs were used as a tool to investigate the relationship between Tregs and IDO in atherosclerosis. IDO1 is considered a major regulator of the immune system due to its ability to deplete Trp from the microenvironment, leading to the activation of general control non-derepressible 2 and the inhibition of basic cellular mechanisms, such as protein synthesis and immune cell division (26, 27). Equally important, the kynurenine pathway can lead to the generation of bioactive Trp metabolites,

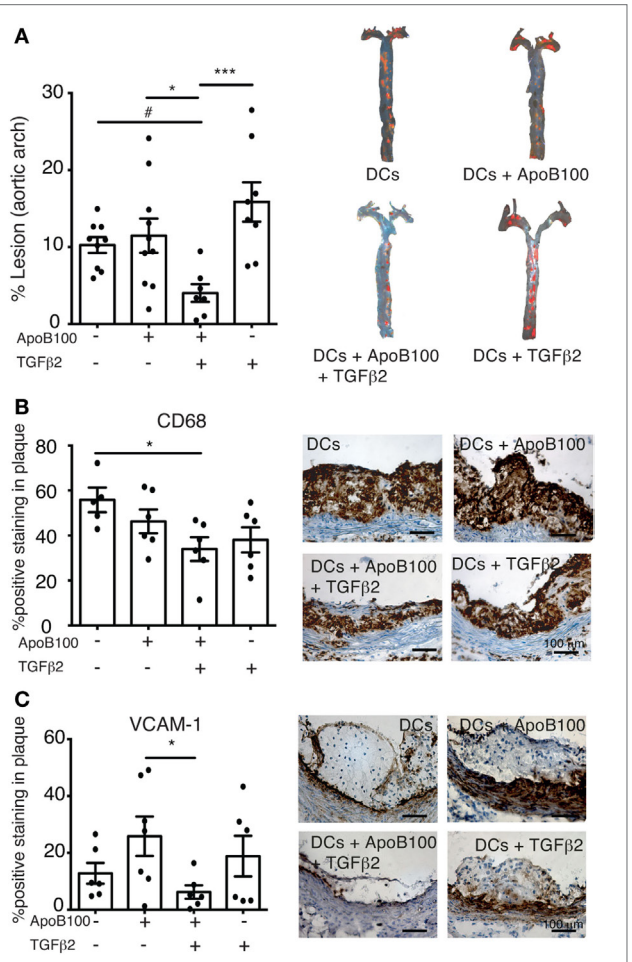

FIGURE 4 | Activation of IDO–regulatory T cell axis reduces atherosclerosis lesions and vascular inflammation. **(A)** Quantitative analysis of atherosclerotic lesion areas in thoracic aortas from mice treated with dendritic cells (DCs) alone ($n = 9$), DCs loaded with apolipoprotein B-100 (ApoB100) ($n = 10$), DCs loaded with ApoB100 and treated with tumor growth factor beta 2 (TGFβ$_2$) ($n = 7$), and DCs treated with TGFβ$_2$ alone ($n = 8$). Right panels: representative images of mouse thoracic aortas showing Sudan IV plaque lipid staining. **(B)** Analysis of CD68-positive cell immunostaining in atherosclerotic plaques from different DC-treated mice. Right panels: representative microphotographs of CD68 immunostaining in atherosclerotic plaques from aortic roots. **(C)** Analysis of vascular cell adhesion molecule 1 (VCAM-1)-positive immunostaining in atherosclerotic plaques from DC-treated mice. Right panels: representative microphotographs of VCAM-1 immunostaining in atherosclerotic plaques from aortic roots. The data are expressed as the mean ± SEM ($^{\#}P = 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

TABLE 1 | Body weight and lipid characteristics of the treated mice.

DC groups	*n*	Body weight (g)	Cholesterol (mg/dL)	Triglycerides (mg/dL)
Untreated	9	27 ± 1.0	1,099 ± 96	818.3 ± 94.2
ApoB100	7	29 ± 1.8	1,297 ± 124	1,087 ± 60.7
ApoB100 + TFGβ$_2$	7	28 ± 1.2	1,338 ± 120	911.3 ± 134.0
TFGβ$_2$	8	27 ± 0.6	1,687 ± 72	1,010 ± 189.7

Data are expressed as the average ± SEM.
ApoB100, apolipoprotein B100; DC, dendritic cell; TGFβ$_2$, tumor growth factor beta 2.

such as 3-hydroxyanthranilic acid (3-HAA), which can directly influence innate and adaptive immune cell inflammatory responses, including Treg induction (28).

Because TGFβ$_2$ is highly expressed in immune-privileged sites and demonstrates potent immunosuppressive effects (29), we hypothesized that treating DCs with this cytokine could yield more Tregs *in vivo*. Indeed, the TGFβ$_2$-induced tolerogenic DCs showed a robust capacity to induce Tregs *in vitro* and *in vivo*, suggesting that TGFβ$_2$ may promote a more potent tolerogenic phenotype in DCs than IL-10 (5).

Regulatory T cells play an essential role in the maintenance of tolerance to self-antigens and suppressing excessive and deleterious immune responses. Similarly, in atherosclerosis, Tregs are associated with many protective functions, including the inhibition of effector T-cell responses (30), suppression of the maturation and immune-stimulatory capacity of DCs (31), modulation of macrophage- and endothelial cell-mediated pro-inflammatory responses (32, 33), and the promotion of proliferation and collagen production by SMCs (34).

While it is well known that Tregs can exert their immunosuppressive effects by secreting anti-inflammatory cytokines (3), a new mechanism has been recently described, the capacity to induce the immunomodulatory enzyme IDO (35). According to various studies, TGFβ or CTLA4 expression on Treg can regulate IDO in DCs and participate in the self-amplification of IDO1 expression and the maintenance of the tolerogenic phenotype of these cells (13, 36, 37). In humans, Treg activity is reduced in RA patients due to the epigenetically mediated downregulation of CTLA4, which leads to reduced IDO1 expression in APCs (14). Interestingly, tolerogenic DC-induced Treg suppression of collagen-induced arthritis was shown to be lost following pre-treatment with the IDO inhibitor 1-MT (38).

Cytotoxic T-lymphocyte associated protein-4 is a co-inhibitory molecule constitutively expressed by Tregs that plays a critical role in peripheral tolerance (39). CTLA4 is homologous to CD28, which binds with a high affinity to CD80 or CD86 and contributes to the IDO-mediated regulatory T-cell generation through pathways that converge on non-canonical NF-κB signaling in APCs (40). While the CTLA4-mediated induction of IDO is well-established in the context of the immune synapse involving T cells and DCs (35), our finding showing that similar mechanisms regulate IDO1 in other cells in the vascular wall is completely novel. Moreover, our data suggest that CD80 rather than CD86 is the major transducer of CTLA4-mediated effects on vascular cells.

Our study shows that IDO1 is predominantly expressed in SMCs, macrophages and endothelial cells in the arterial wall. Expectedly, in our *in vitro* assays using these cells, IDO1 expression and Trp degradation increased upon IFNγ stimulation. Remarkably, CTLA4 substantially potentiated the IFNγ effects and increases IDO activity in these cells, particularly in the SMCs. These data are in line with previous studies indicating that IDO expression in vascular SMCs contributes to the enhancement of the natural resistance of the vascular wall to inflammation (41). In fact, we have previously shown that IDO-dependent Trp metabolism can influence the expression of VCAM-1 in human coronary artery SMCs *in vitro* and tunica media SMCs *in vivo* in *Apoe*$^{-/-}$ mice. Interestingly, in both cases, the

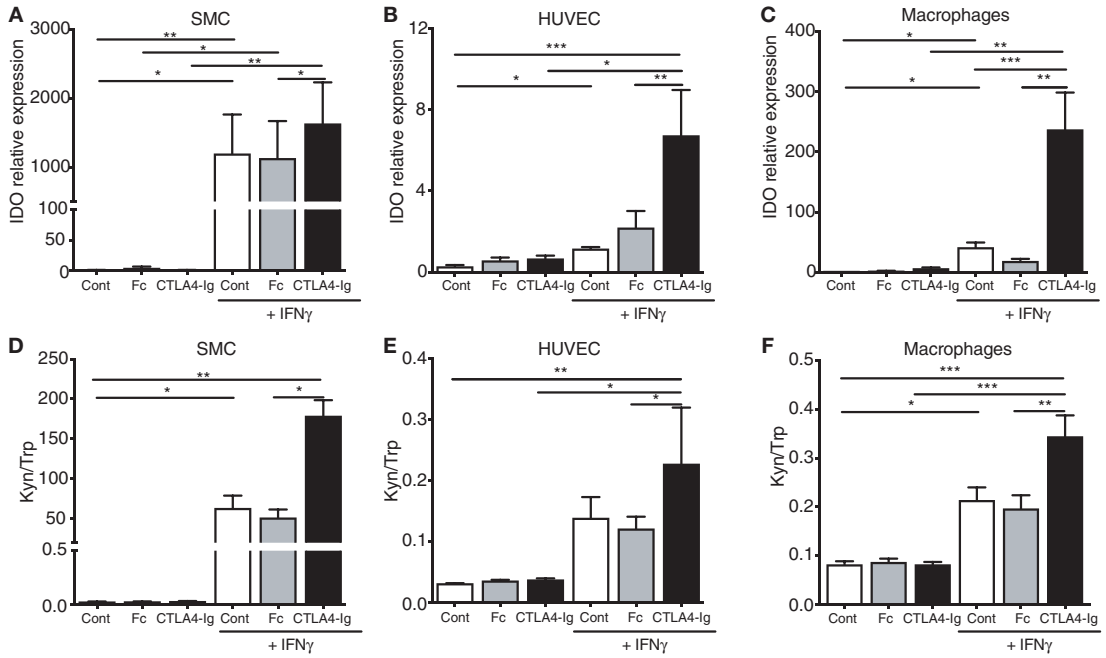

FIGURE 5 | Cytotoxic T-lymphocyte associated protein-4 (CTLA4)-Ig enhances indoleamine 2,3-dioxygenase-1 mRNA expression and enzymatic activity in human smooth muscle cells (SMCs), endothelial cells, and macrophages. **(A–C)** IDO mRNA expression in human **(A)** SMCs, **(B)** human umbilical vein endothelial cells (HUVECs), and **(C)** macrophages cultured for 24 h with or without 400 U/mL IFNγ, washed and subsequently treated with CTLA4-Ig, isotype Fc, or medium alone. **(D–F)** Kynurenine to tryptophan ratio (Kyn/Trp) in the supernatants of human **(D)** SMCs, **(E)** HUVECs, and **(F)** macrophages cultured for 24 h with or without 400 U/mL IFNγ, washed and subsequently treated with CTLA4-Ig, Isotype Fc, or medium alone. The SMC results represent pooled data from five independent experiments (triplicate wells) using cells from two different donors. The HUVEC and macrophage results represent pooled data from five independent experiments each (triplicate wells) using cells from five different donors. Unstimulated cells were used as a control. The data are expressed as the mean ± SEM ($^{\#}P = 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

VCAM-1 upregulation could be reversed by the administration of 3-HAA (11).

Our data suggest that Treg interactions with SMCs, macrophages and endothelial cells may not be restricted to soluble factors, e.g., TGFβ that regulate SMC proliferation and collagen production (42). Speculatively, the known role of IDO in the differentiation of Tregs (40) suggests that SMCs could play a role in Treg-induced "infectious tolerance" in the artery wall.

Although macrophages show a less pronounced increase in IDO activity upon CTLA4 ligation, they are the most abundant cell type in the plaque and their contribution to modulation could also be relevant. It has been shown that monocyte-derived macrophages can suppress T-cell proliferation *in vitro via* the rapid and selective degradation of Trp by IDO1 (43). Thus, the high rates of Trp degradation and the formation of bioactive Trp metabolites by SMCs, macrophages, and endothelial cells could constitute an important feedback loop that stabilizes and even enhances the effects of Tregs in the vessel wall.

The administration of the CTLA4-Ig fusion protein to hypercholesterolemic mice substantially reduces atherosclerosis, whereas a CTLA4 blockade accelerates disease (44). While it was not explored in the latter study, our data suggest that the IDO1 induction by CTLA4 could be an important atheroprotective mechanism induced by the treatment. Interestingly, CTLA4-Ig (abatacept) is currently under investigation as a first-line biologic for the treatment of autoimmune diseases, such as RA (45). Based on our findings, CTLA4-Ig emerges as a potential candidate for the prevention of CVDs due not only to its inhibitory effects on different immune cells but also to its IDO induction capabilities.

In conclusion, we show that the triggering of the "Treg–IDO axis" in the vascular wall generates a tolerance-sustained milieu characterized by reduced vascular inflammation and atherosclerotic lesion formation. Based on our study, strategies that reinforce the IDO1 pathway in a cell- or tissue-specific manner, such as the use of tolerogenic DCs and the induction of antigen-specific Tregs, have high therapeutic potential against atherosclerotic CVDs.

ETHICS STATEMENT

All animal experiments were conducted according to the guidelines of Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and approved by the Stockholm Norra regional ethical board.

AUTHOR CONTRIBUTIONS

DK and GH designed the project and supervised the research. MF, KP, RB, BS, MM, DJ, AH, and DK performed the experiments.

MF, KP, RB, BS, and MM analyzed and interpreted data. GH contributed with the critical interpretation of data and revision of the manuscript. MF and DK wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00950/full#supplementary-material.

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Conflict of Interest Statement: GH and AH hold patents on the use of tolerogenic DCs for the prevention and treatment of atherosclerosis. DK and GH hold patents on the use of 3-HAA for the prevention and treatment of hyperlipidemia and its complications. The remaining authors have no disclosures to report.

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Non-Invasive Multiphoton Imaging of Islets Transplanted Into the Pinna of the NOD Mouse Ear Reveals the Immediate Effect of Anti-CD3 Treatment in Autoimmune Diabetes

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We present a novel and readily accessible method facilitating cellular time-resolved imaging of transplanted pancreatic islets. Grafting of islets to the mouse ear pinna allows non-invasive, *in vivo* longitudinal imaging of events in the islets and enables improved acquisition of experimental data and use of fewer experimental animals than is possible using invasive techniques, as the same mouse can be assessed for the presence of islet infiltrating cells before and after immune intervention. We have applied this method to investigating therapeutic protection of beta cells through the well-established use of anti-CD3 injection, and have acquired unprecedented data on the nature and rapidity of the effect on the islet infiltrating T cells. We demonstrate that infusion of anti-CD3 antibody leads to immediate effects on islet infiltrating T cells in islet grafts in the pinna of the ear, and causes them to increase their speed and displacement within 20 min of infusion. This technique overcomes several technical challenges associated with intra-vital imaging of pancreatic immune responses and facilitates routine study of beta islet cell development, differentiation, and function in health and disease.

Keywords: type 1 diabetes, islets, multiphoton, imaging, immunotherapy, anti-CD3, T cell, autoimmunity

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INTRODUCTION

Treatment with anti-CD3 results in reversal of established diabetes in NOD mice (1) in a TGFβ-dependent manner (2), involving combination of early and long lasting mechanisms, including clearance of infiltrating cells from the islets, and a time limited reduction in the numbers of circulating T cells (3, 4). Previous work has established that anti-CD3 treatment leads to resolution of the lymphocytic infiltrates within 24–48 h (5), but it remains unclear whether this resolution is due to death of the islet-reactive cells (6) or to a change in their behavior (7–9). We have used a novel method for imaging infiltrating immune cells in islets transplanted into the pinna of the mouse ear to clarify the intra-islet events directly following anti-CD3 administration.

Disease progression in experimental type 1 diabetes is usually studied by excising the pancreata from experimental mice and analyzing the cell content, which provides a snapshot image of the inflammatory status of the islets. However, this precludes an appreciation of the dynamic temporal nature of disease processes. For studying the contribution of cellular behavior in deployment of immune effector functions, high-resolution real-time imaging must be performed (10). Multiphoton laser scanning microscopy of isolated islets from RIP-OVA mice has been used to follow the behavior of inflammatory cells infiltrating the islet. This has revealed that stable $CD8^+$ T cell/APC interactions only occur early in islet invasion and are associated with induction of IFNγ production (11). Further development of intravital techniques confirmed this behavior in intact, *in situ* pancreas in the NOD mouse (12). However, the asynchronous infiltration of islets poses challenges not only for pooled islet analysis but also for longitudinal studies. Differences in disease progression between individual mice has meant that group sizes have had to be large (at least five mice per group) to estimate the effects of any treatment protocols on pancreatic events.

A way of solving the problem of continuity of observation while reducing the number of experimental mice is to visualize events in the islet through imaging, without having to remove the islet from the mouse (10). Although several groups have demonstrated feasibility of cellular imaging approaches, through surgical exposure of the pancreas (13) or an islet graft under the kidney capsule (14), the invasiveness of the procedure makes longitudinal studies impractical. Although technically challenging, insertion of an imaging window can allow visualization of islets transplanted to the kidney capsule thus facilitating repeated imaging over time (15). A major advance in permitting longitudinal studies of islet biology has been in the imaging of islets transplanted into the anterior chamber of the eye (ACE) (16), capturing images of islet vasculature (16), insulin resistance (17), beta cell mass (18, 19), as well as immune cell infiltration (19, 20). However, while this method has clear advantages for longitudinal study of islet biology, relevant immunological processes may be strongly affected by the immune privilege given by the ACE environment (21–23). Anterior chamber associated immune deviation is believed to be caused at least in part by the high levels of TGF beta in the vitreous fluid (21), and this may well affect the immune reactions we wish to record (24–26). As our interest is to monitor effects of immunomodulatory treatments, these concerns led us to examine non-invasive imaging of islets in a different site, in the pinna of the ear. The mouse ear pinna is easily accessible by various imaging modalities and compatible with studies of normal immune functions. Indeed, we have previously demonstrated longitudinal *in vivo* imaging and normal immune function (antigen drainage, lymphocyte priming, and recirculation) of lymph nodes engrafted in the pinna (27). This site has also been shown to support engraftment of a variety of different tissues including heart, thymus, kidney, adrenal gland, and spleen which not only survive, but continue to function in an organ specific way (27–30).

To test whether islets grafted into the pinna of the ear can serve as an easily accessible site for non-invasive imaging of effects of treatments aimed to decrease islet inflammation, we determined that islets can indeed become established in the pinna, and that they continue to produce insulin and glucagon therein. We then investigated whether we could use intravital imaging to further investigate the nature of the resolution of islet immune infiltration caused by administration of non-FcR binding anti-CD3 antibody, which is a mouse equivalent to Otelixizumab which has been used in clinical trials in patients (31, 32). Our data demonstrate that non-invasive imaging of islet grafts in the pinna can detect the clearance of infiltrates seen with invasive methods. Our results also show that in addition to any cell death occurring after injection of anti-CD3, there is an immediate increase in T cell motility after administration, indicating reduced interaction with targets and APC which can have profound effects on T cell tolerance (33, 34).

This technique enables immunological processes to be followed in individual animals over several time points with minimal discomfort, using fewer laboratory animals and allowing for more accurate data acquisition.

MATERIALS AND METHODS

Mice and Diabetes Detection

NOD, NOD-*scid* NOD, NOD-hCD2-GFP (35), Kaede-C57BL/6 (36) and NOD Foxp3-GFP mice (37), hCD2-GFP (38), and CD11cYFP (39) were bred and maintained under specific pathogen-free barrier conditions. C57BL/6 mice were purchased from Charles River. This study was carried out in accordance with the recommendations of the Animals (Scientific Procedures) Act. The protocols were approved by the UK Home Office and performed under the Project Licences of PG, KO, and MW. Mice were housed in individually ventilated cages with free access to standard chow and water. The facility is kept on a 12 h light, 12 h dark cycle. The humane endpoints for these experiments specify that any mouse that loses more than 15% of its body weight (compared with healthy littermates), or in other ways looks unwell and likely to exceed the Home Office standard of moderate severity must be culled. However, no mice used in this study required early culling. At the end of the experiments, mice were culled using a CO_2-chamber followed by dislocation of the neck. In cases where we were harvesting islets for transplantation it was important to maintain an intact bile duct, and death was instead confirmed through palpation of the chest to assess the absence of a heart beat followed by confirmation of cessation of blood flow. Diabetes was detected using Diastix reagent strips (Bayer Diagnostics, Basingstoke, UK) and confirmed by a blood glucose measurement of >13.3 mM, using a Breeze2 blood glucose meter (Bayer). All animal work was conducted under UK Home Office project licence regulations after approval by the Ethical Review Committees of the University of Cambridge and Glasgow, respectively.

Islet Isolation and Transplantation

Pancreatic islets were isolated through inflation of the pancreas *via* the bile duct (40), and islets were prepared for transplantation by insertion into polyethylene tubing as previously described (41). Islets were prepared from mice ranging from

6 to 14 weeks of age, and recipient mice were between 10 and 12 weeks of age. Analgesic (Temgesic, Reckitt Benckiser) was administered s.c. in the scruff prior to anesthesia (Isoflurane, Abbott Laboratories). Ears were immobilized using double sided sticky tape for creation of a pocket on the ventral side of the pinna using forceps. A single piece of tubing containing the islets was inserted into the pocket, and approximately 50 islets deposited into the space. The incision was then closed with veterinary-grade glue (Vetbond, 3M).

Aglycosyl Anti-CD3 Antibody

The non-Fc receptor binding anti-mouse CD3 antibody (agly anti-CD3) was generated through genetic engineering in the Waldmann Laboratory, University of Oxford as previously described (9). The antibody that recognizes human CD3 (Otelixizumab) was used as a non-binding isotype control.

Multiphoton Laser Scanning Microscopy

Intravital microscopy studies were carried out using a Zeiss LSM7 MP system or a Leica SP8 MP system (Molecular Immunology Unit, University of Cambridge) equipped with a tuneable Titanium: sapphire solid-state two-photon excitation source (4W, Chameleon Ultra II, Coherent Laser Group) coupled to an Optical Parametric Oscillator (Chameleon Compact OPO; Coherent). Movies were acquired for 20–30 min with an X–Y pixel resolution of 512 × 512 in 2 μm Z increments. 3D tracking was performed using Volocity 6.1.1 (Perkin Elmer, Cambridge, UK) or Imaris (Bitplane). Cells were identified using intensity thresholding and object volume. Track plots are included to demonstrate the actual migration of cells relative to their point of origin. To calculate displacement rate, we first calculated the straight line distance between the point of origin and the point of termination of a cell track and then divided the distance by the duration of the cell track in minutes. Maximum speed is presented in micrometer per minutes. Meandering index (also known as confinement ratio or chemotactic index) is a ratio defining track straightness (ratio of displacement of the cell to the total length of the track) where 0 represents a highly confined cell that returns to its starting position, and 1 being a cell traveling in a completely straight line. Mice were anaesthetized using Domitor (50 mg/kg Ketamine and 0.5 mg/kg Medetomidine i.p.) and placed on a heated stage. The ear of interest was mounted on a purpose-built stand with the use of veterinary-grade glue (Vetbond; 3M), enabling warming of tissue throughout the experiment (maintained at 37°C). To allow repeated imaging sessions, veterinary glue was dissolved with 70% ethanol, and mice given Antisedan (1 mg/kg Atipamezole s.c.) for reversal of the Domitor anesthesia. Blood vessels were visualized by i.v. injection of non-targeted quantum dots (Qtracker 655, Life Technologies). SYTOX was used to image cell death *in situ*. 10 μl of 10 μM SYTOX orange (Molecular Probes, Thermo Fischer, UK) was injected into the ear pinna 10 min prior to imaging to allow visualization of dead cells (42). To image naïve T cells in lymph nodes, DsRed expressing $CD4^+$T cells from unimmunized mice were magnetically sorted from hCD2-DsRed animals using mouse $CD4^+$T cell isolation kits (Miltenyi Biotec, Surrey, UK). 2–3 × 10^6 purified T cells were then adoptively transferred into naïve CD11cYFP recipient mice. Popliteal LNs were excised 24 h later for imaging. LNs were maintained by being continuously bathed in warmed (37°C), oxygenated CO_2 independent medium (43). For studies of the effects of aglycosylated anti-CD3, 20 μg of active antibody or isotype control (9) was injected i.v. as indicated. Imaging was interrupted for injection of antibody, and started no more than 5 min after injection. The results achieved in the multiphoton imaging facility at the University of Glasgow were also replicated in the facility at the Babraham Institute, Cambridge. A 1:1 mixture of Hypnorm (25 mg/kg) and Hypnovel (12.5 mg/kg) injected i.p. was used to induce anesthesia during imaging at the Babraham Institute. Movies were acquired for the indicated time with an X–Y pixel resolution of 512 × 512 in 6.23 μm Z increments and a total depth of 62.3–68.53 μm with a 30 s frame interval.

For imaging of islets in the pancreas at the University of Cambridge, mice were anesthetized using 3% isoflurane, the pancreas externalized, submerged in surgical saline solution, and immobilized under a cover slip and the mouse placed in a 37°C environmental chamber for imaging. Islets were imaged using a 20× water immersion objective for up to 1 h per movie. Movies were acquired with an X–Y pixel resolution of 512 × 512 in 2 μm Z increments and a total depth of 54–67 μm with a 40 s frame interval.

Immunofluorescence

Pancreata and graft-bearing pinnae were fixed in 4% PFA for 72 h, dehydrated in sucrose, and mounted in OCT. 10 μm sections were cut on a Leica cryostat and placed on Polysine slides (VWR), air dried, and fixed again for 10 min in acetone. Sections were stained using guinea pig-anti-insulin antibody (DAKO) at a 1:40 dilution and detected with anti-guinea pig Alexa 546 (Molecular Probes). Rabbit anti-human glucagon (Millipore) was used at a 1:50 dilution and detected with anti-rabbit Alexa 488 (Molecular Probes). Anti-mouse CD4 (BD) was detected with anti-rat Alexa 488 (Molecular Probes). Nuclei were visualized with DAPI (Molecular Probes). The sections were viewed using either a Zeiss Axioskop 2 or LSM700 Confocal (Zeiss) and processed using Zen software.

Flow Cytometry

Cells were prepared from pancreas and islet grafts in pinna through manual dispersion of the tissue followed by digestion for 10 min at 37°C in 0.5% collagenase P solution (Sigma). Cell suspensions from lymph nodes were prepared through dispersion between glass slides. All cell preparations were resuspended in FACS buffer (PBS with 0.5% BSA), filtered through 30 μm Celltrics filters (Partec), incubated with Fc-block (eBioscience) and then stained with CD19 Alexa647 (eBioscience), washed, and resuspended in PBS containing 5% 7AAD (BD Bioscience). Data were collected on a Cyan Cell Cytometer (DAKO) and the data analyzed using FlowJo (Tree Star Inc.).

Statistical Analysis

Differences between two groups were assessed with a non-parametric Mann–Whitney test, and for multiple comparisons

we used the non-parametric Kruskal–Wallis test with a Dunn's post-test.

RESULTS

The Pinna of the Ear as a Site for Islet Transplantation

To demonstrate the feasibility of the pinna as a suitable graft site for islets, we grafted C57BL/6 islets into the pinna of syngeneic C57BL/6 recipient mice. These mice have no pre-existing autoimmunity to islets, and can accept syngeneic islet grafts under the kidney capsule (44). Islets of Langerhans were isolated (40) and prepared for transplantation as previously described (41). The islets were then deposited into a small pocket in the skin on the ventral side of the pinna (**Figure 1A**). Grafted islets could be detected in the ear pinna up to 12 weeks post-transplantation and were seen to produce insulin (**Figure 1B**) (later time points were not assessed). Indeed, insulin and glucagon producing cells were readily detected in the islets in the pinna (**Figures 1C,D**). Although the gross architecture of the islets grafted into the pinna appeared normal, glucagon-producing alpha cells were found throughout the islet rather than along the perimeter edge as seen in islets directly from the pancreas (**Figure 1E**).

To determine whether the pinna would be feasible for non-invasive imaging, we transplanted fluorescent islets from Kaede C57BL/6 mice (36) into C57BL/6 recipients (**Figure 1F**). The fluorescent islets were accepted, and fluorescence could be detected using whole body imaging (**Figure 1G**) and multiphoton imaging (**Figure 1H**; Movie S1 in Supplementary Material). The grafted pinnae were removed after imaging and stained for insulin, again demonstrating the presence of insulin-producing cells in the grafted islets (**Figures 1I–K**). Thus, islets grafted to the ear pinna can survive, are functional, and readily amenable to non-invasive imaging.

Use of Islet Grafts in the Pinna to Assess Immune Infiltration

Given that the grafted tissue can be imaged non-invasively, we sought to determine the potential for monitoring the T cell infiltrate that mediates β cell destruction in NOD mice. We grafted NOD-*scid* islets into the pinna of CD2-GFP NOD recipients (**Figure 2A**), allowing tracking of T cell responses where there is existing immune reactivity to islet antigens and established pancreatic immune infiltration. Ten days after transplantation, foci of GFP fluorescence could be detected, as well as individual immune cells in the infiltrate whose movements could be tracked (**Figure 2B**, top panel; Movie S2 in Supplementary Material). Imaging of naïve T cells in lymph node (**Figure 2B**, lower panel) demonstrates that the cells infiltrating the transplanted islets behave more like activated cells engaging a target (45), displaying a lower velocity and displacement than naive cells (**Figure 2B**, plotted in **Figure 2C**). Insulin staining of the islet graft after fixation of the pinna demonstrates the presence of insulin producing cells in the pinna, with considerable presence of infiltrating immune cells (**Figure 2D**), which corresponds to the presence of advanced infiltration of the islets in the pancreas of the same graft recipient (**Figure 2E**, right panel). Using an injectable cell death dye, Sytox, we could also monitor cell death in the grafted pinna while imaging (Movie S3 in Supplementary Material). Use of a different transgenic recipient strain in which all Foxp3 expressing cells also express GFP, enabled imaging of the presence and behavior of Foxp3$^+$ Treg cells in the grafts (Movie S4 in Supplementary Material). These data demonstrate that infiltration of lymphocytes into the islet graft can be monitored and quantified *in vivo* without need of surgical exposure of the tissue for multiphoton imaging.

Determination of Effects of Aglycosyl Anti-CD3 Treatment on Islet Grafts in the Pinna

To assess whether immune infiltrate into islets grafted into the pinna reacted in a similar way to established immunotherapy as infiltrates in the pancreas, we assessed the response to injection of a non-lytic aglycosyl anti-CD3, which can prevent rejection of allografts (46) and reverse overt diabetes through loss of islet infiltrating cells (1, 6). We grafted NOD-*scid* islets into the pinna of CD2-GFP NOD mice on day 0, and imaged infiltration on day 7 (**Figure 3A**). After an initial period of imaging (**Figure 3B**, left hand panel; Movie S5 in Supplementary Material), first 20 μg of isotype antibody was injected i.v. (**Figure 3B**, middle panel; Movie S6 in Supplementary Material) and then 20 μg of agly CD3 was injected i.v., and the same area of the graft imaged again to monitor any change in the behavior of infiltrating cells (**Figure 3B**, right-hand panel; Movie S7 in Supplementary Material). Imaging was interrupted for injection of antibody and started no more than 5 min after injection. Analysis of the movement of the cells before and after injection of agly CD3 (**Figure 3C**) demonstrated a marked increase in speed and displacement rate after the injection of anti-CD3 but not isotype antibody, indicating that the active antibody had an immediate effect on the islet reversing the T cell arrest required for TCR binding and T cell activation (**Figure 3D**) (47). The injection of isotype antibody induced modest increase in cell displacement in some experiments, but not to the same extent as the active anti-CD3 antibody (**Figure 3C**). The observed increase in T cell speed and displacement was confirmed by identical results recorded when performing the procedure and imaging in the facilities at the University of Glasgow (Figure S1 and Movies S8 and S9 in Supplementary Material). When we injected 20 μg of agly CD3 on days 7, 8, and 9, and imaged again on day 10, we found that the infiltrates in the treated mouse, which had abundant infiltrates on day 7, were dispersed after 3 days of agly anti-CD3 treatment (Figure S2 in Supplementary Material), mirroring the effect of this treatment regimen in islets in the pancreas. Staining of sections of grafted pinnae and pancreata of islet graft recipient mice either receiving agly anti-CD3 (**Figure 3E**, central panels) or not (**Figure 3E**, left-hand panels), also demonstrated the reduction of infiltrating T cells both in islets in the pinna (**Figure 3E**, top panels) and pancreas (**Figure 3E**, bottom panels) after treatment. These results were further supported by assessment of infiltrating cells *via* flow cytometry of excised grafts from the pinna (Figure S2 in

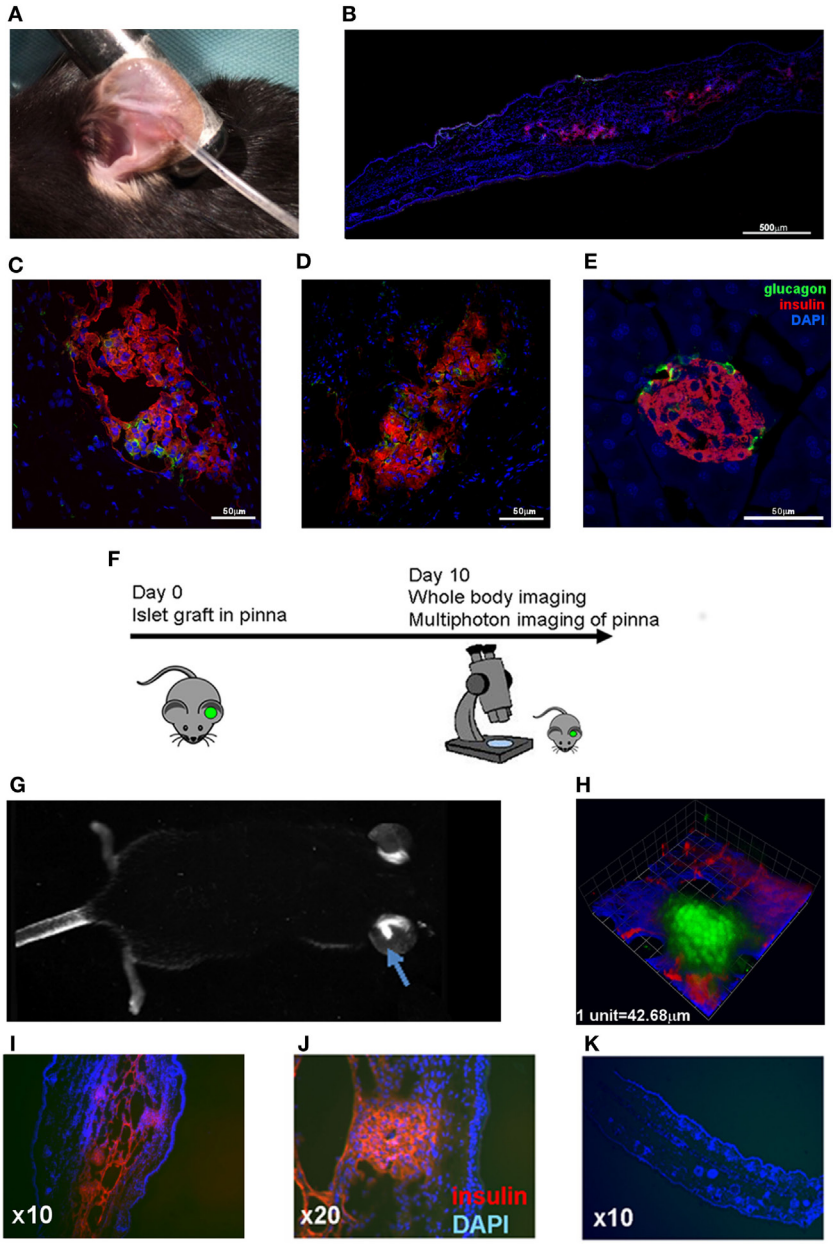

FIGURE 1 | Pancreatic islets grafted into the pinna of the ear retain capacity to produce insulin and can be used for non-invasive imaging. **(A)** C57BL/6 islets were grafted into the pinna of syngeneic C57BL/6 recipients. **(B)** 12 weeks later, islets were readily detected in the pinna, and higher magnification revealed glucagon positive cells (green) among the insulin positive beta cells (red) **(C,D)**. The same staining was performed on islets in the pancreas of the graft recipient **(E)**. **(F)** Islets isolated from a Kaede transgenic mice were grafted into the pinna of syngeneic C57BL/6 mice. On day 10, the graft recipient was anaesthetized and analyzed with whole body imaging and multiphoton microscopy. **(G)** On day 10, green fluorescent islets could be detected in the pinna using whole body imaging. An arrow indicates the fluorescent mass in the grafted ear. **(H)** The green fluorescent grafted islets could also be detected with multiphoton imaging (3D rendering of a Z-stack capture). **(I,J)** The graft bearing pinnae were fixed and stained to check for insulin production (red), and compared with a cross section of a control pinna **(K)**. The selected images are representative of results from groups containing at least three mice.

Supplementary Material), showing a decrease in the presence of GFP positive T cells in mice treated with agly anti-CD3 compared with isotype antibody treated controls. We confirmed that the observed change in behavior in the islet infiltrating cells in the pinna is representative of the events in the native pancreas after injection of agly anti-CD3 by direct imaging of the pancreas. We found that islet infiltrating T cells increase their speed and displacement after injection of agly anti-CD3 antibody in a similar way as the infiltrates in the pinna islet grafts (**Figure 4**; Movies S10 and S11 in Supplementary Material).

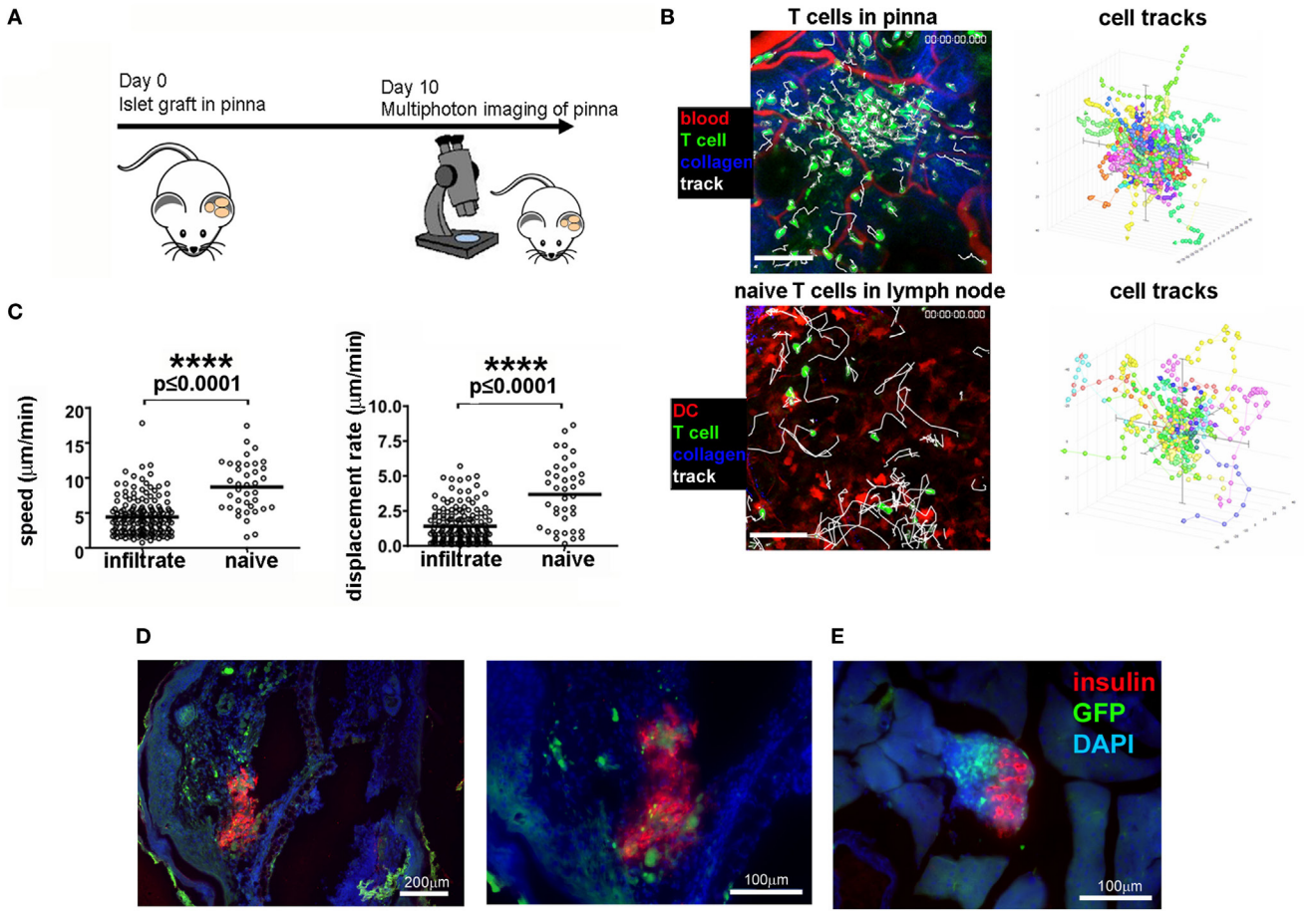

FIGURE 2 | Imaging of T cell infiltration in NOD-*scid* islets transplanted into the pinna of the ear of a CD2-GFP NOD recipient. **(A)** On day 0, islets were isolated from a NOD-*scid* mouse and grafted into the pinna of a CD2-GFP NOD mouse. On day 10, the graft recipient was anesthetized and the graft imaged using multiphoton microscopy. **(B)** Multiphoton imaging showing either islet-infiltrating GFP$^+$ T cells (green) around a grafted islet in a 12-week old NOD recipient intact ear pinna (top panels), or naïve T cells moving in a lymph node (bottom panels). Scale bars represent 100 μm. The right-hand side flower plots demonstrate the movement of the T cells during imaging **(B)**, presented in graph form in **(C)**. After imaging, the pinna **(D)** and pancreas **(E)** were fixed and stained for insulin (red) and DNA stain DAPI (blue). T cells can be identified by green GFP signal. The selected images are representative of results from groups containing ≥ three mice.

DISCUSSION

By engrafting islets to the ear pinna, we have circumvented issues of immune privilege and invasiveness to allow study of immune mechanisms mediating beta cell destruction and therapeutic efficacy. It has been shown that the pinna of the ear is a suitable site for transplantation of a range of tissues (28–30) and for multiphoton imaging (27), supporting conventional lymphatic drainage, revascularization of the grafted tissue, and normal lymphocyte responses (27, 28). Furthermore, the pinna represents an easily accessible tissue for the grafting procedure as well as for microscope access and minimization of drift during imaging, making this an ideal site for longitudinal *in vivo* cellular imaging studies. We show that anti-CD3 treatment leads to an immediate increase in movement and displacement of infiltrating immune cells in islets in the pancreas, and that we can use imaging of islets transplanted into the pinna to image this process in a less invasive way. We present unprecedented data on the nature and rapidity of the effect on the islet infiltrating T cells.

In the more than 20 years since Lucienne Chatenoud's pioneering experiments demonstrating reversal of diabetes in experimental mice (1), the mechanisms through which the anti-CD3 antibodies exert their effect have been gradually clarified. They work through a wide array immediate and long-term effects, including downregulation of the CD3 signaling complex (48), induction of anergy and apoptosis (49, 50), and increased regulation through elevated levels of TGF beta (24) and an expanded Treg pool (6, 51), as reviewed in Ref. (3, 4). A compelling suggestion is that the observed increase in the Foxp3$^+$ Treg population after anti-CD3 treatment is due to a preferential depletion of activated T cells (6), a theory which has been supported by studies using OVA specific cells (6, 46). Our studies utilizing GFP labeled islet antigen-specific Th1 cells for tracking cell fate, while replicating the finding of a retained Treg pool, did not, in this model, find a preferential depletion of islet-specific effector T cells by anti-CD3 treatment (9). Instead we found an induction of anergy accompanied by an increase in PD-1 expression in this cell subset, which prevented further activation of islet antigen-presenting APC.

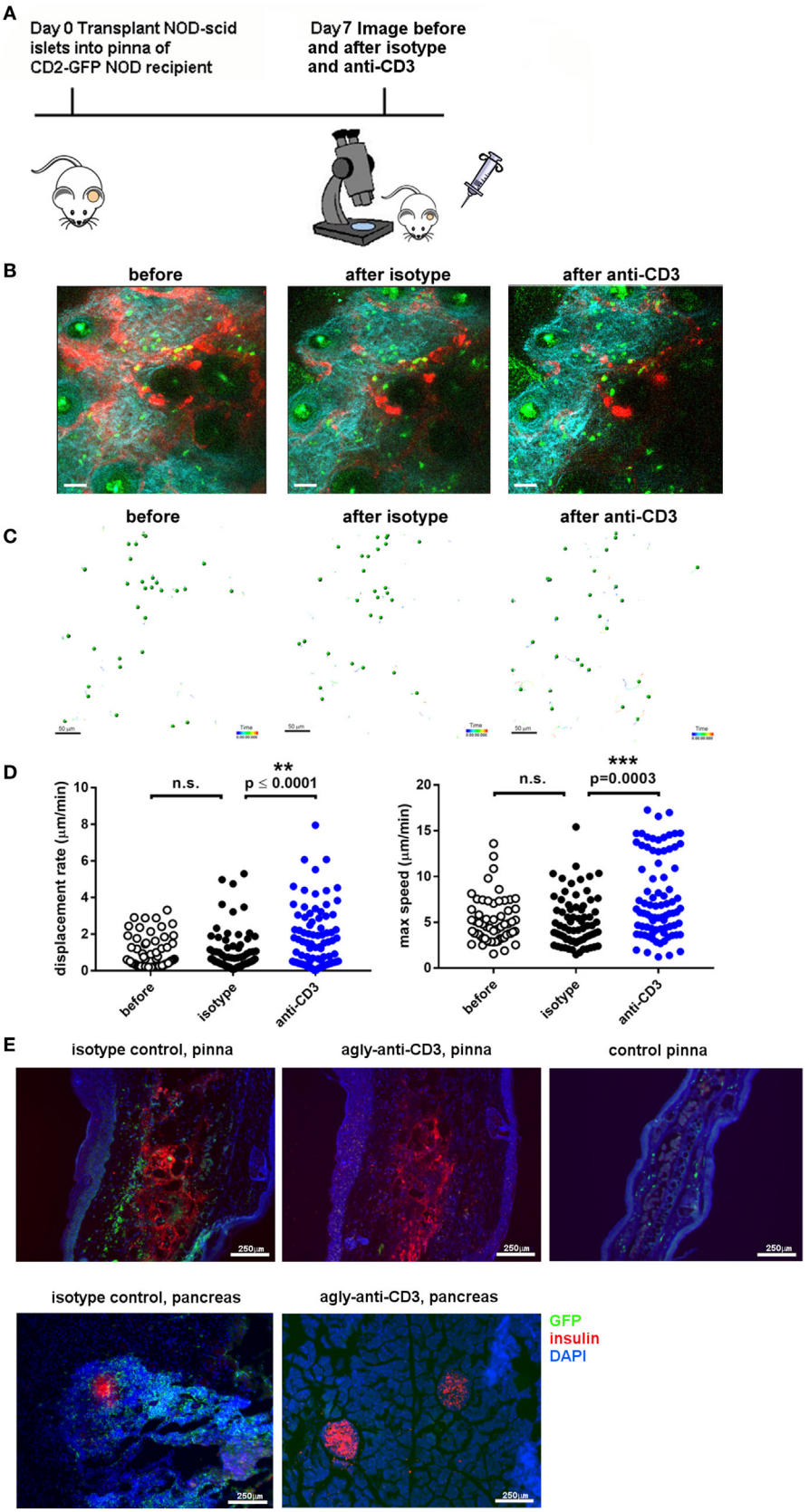

FIGURE 3 | Continued

FIGURE 3 | Imaging of T cell infiltration in NOD-*scid* islets transplanted into the pinna of the ear of a CD2-GFP NOD recipient before and after administration of agly anti-CD3. **(A)** On day 0, islets were isolated from a NOD-*scid* mouse and grafted into the pinna of a CD2-GFP NOD mouse. On day 7, the graft recipient was anesthetized and the graft imaged using multiphoton microscopy. Then 20 µg of first isotype control antibody and then agly anti-CD3 was administered i.v. and the graft imaged again. **(B)** Still images from acquired Z-stack longitudinal multiphoton imaging showing islet-infiltrating GFP$^+$ T cells (green) moving around a grafted islet in a 12-week-old NOD recipient before injection [**(B)**, left panel], after injection of isotype control antibody [**(B)**, center panel], and after injection of aglycosyl anti-CD3 [**(B)**, right panel]. The white bar represents 50 µm. Red = blood vessels, blue = collagen (secondary harmonic signal), green = GFP. **(C)** Depiction of the individual tracks of infiltrating cells during the imaging period in the indicated conditions. **(D)** Plotting of the recorded displacement rate (left) and max speed (right) of islet infiltrating cells before and after administration of isotype antibody or aglycosyl anti-CD3. The data are representative of five independent experiments and differences between groups were analyzed using a non-parametric Kruskal–Wallis test with a Dunn's post-test for multiple comparisons. **(E)** Section staining of pinnae [**(E)**, top panels] and pancreas [**(E)**, bottom panels] from isotype control injected graft recipients [**(E)**, left-hand panels] and aglycosyl anti-CD3 injected [**(E)**, middle panels] or control pinna (top right panel). Red = insulin, blue = DAPI, green = GFP.

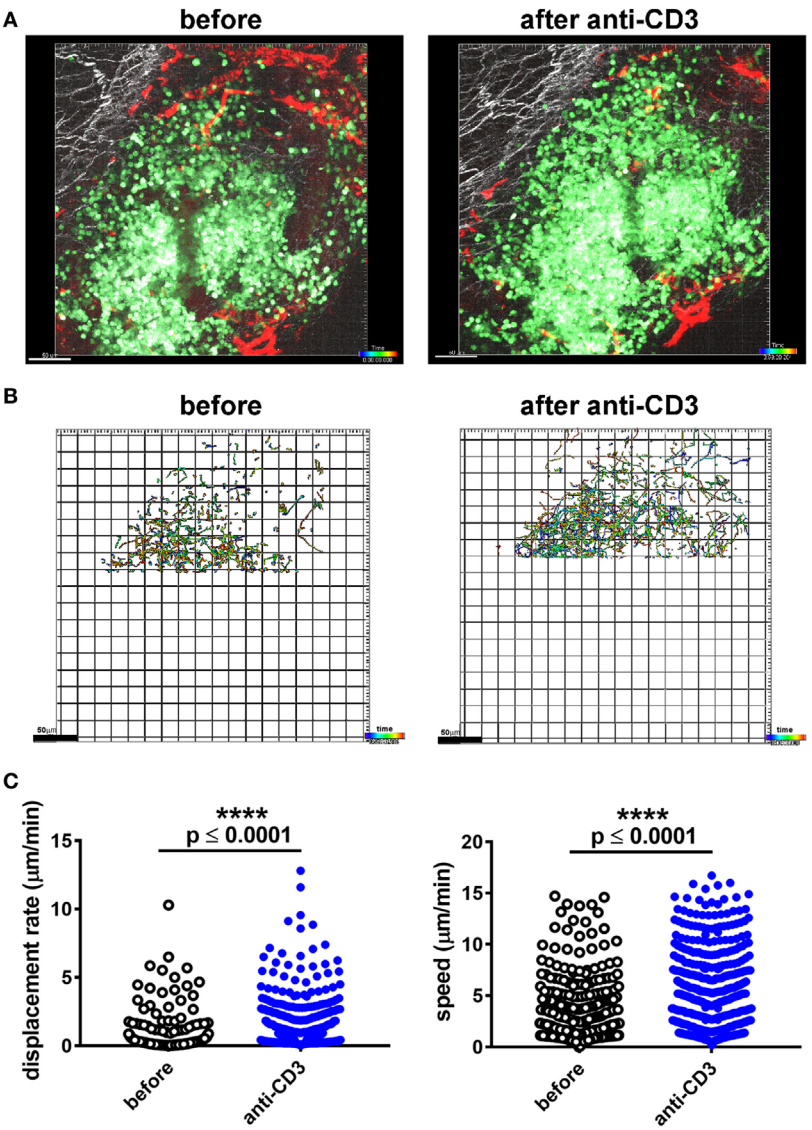

FIGURE 4 | Imaging of T cell infiltration in a pancreatic CD2-GFP NOD islet before and after administration of agly anti-CD3. **(A)** The mouse was anesthetised, the pancreas externalized, and mounted for multiphoton microscopy imaging. The infiltrated islet was imaged before and after i.v. administration of 20 µg of agly anti-CD3. Still image from acquired Z-stack longitudinal multiphoton imaging showing islet-infiltrating GFP$^+$ T cells (green) moving around an islet in a 12-week old NOD recipient before injection [**(A)**, left panel], and after injection of aglycosyl anti-CD3 [**(A)**, right panel]. The white bar represents 50 µm. Red = blood vessels, blue = collagen (secondary harmonic signal), green = GFP. **(B)** Depiction of the individual tracks of infiltrating cells during the imaging period (59 min and 20 s) in the indicated conditions. Data were only acquired from the top part of the capture, as the infiltrates in the lower parts were too dense to track. **(C)** Plotting of the recorded displacement (left) and speed (right) of islet infiltrating cells before and after administration of aglycosyl anti-CD3. The difference between groups was assessed using a non-parametric Mann–Whitney test, and the data are representative of three independent experiments.

Induced anergy in effector T cells is often referred to as exhaustion (52) as it shares many of the same markers, importantly high expression of PD-1. There is an accumulation of $CD8^+$ T cells with an exhausted phenotype in type 1 diabetes patients that respond well to Teplizumab treatment (as measured through preservation of c-peptide production) (53, 54). Studies in islet allograft models also indicate induction of anergy and upregulation of PD-1 on both graft infiltrating $CD8^+$ T cells (55) and $CD4^+$ T cells (8) after anti-CD3 treatment, suggesting that induction of anergy in the effector population rather than expansion of the Treg pool is what affords long-term tolerance after anti-CD3 treatment (8). The requirement for effects on already primed cells is also supported by studies of the importance of timing of the treatment. Anti-CD3 treatment is only effective if administered once the mice have become diabetic, and not before (5). The effects of anti-CD3 treatment on tolerance to transplanted tissue is also highly dependent on the timing of administration (46, 56), with treatment coinciding with the priming of allo-reactive immune responses (3–7 days after transplant) affording long-term graft survival due to preferential activation of tolerogenic pathways (7).

It is well established that recently diagnosed diabetic NOD mice revert to euglycemia within 24–48 h after anti-CD3 treatment (5). We have used non-invasive imaging of islets transplanted into the pinna of the ear to assess just how quickly the effects of the anti-CD3 treatment can be observed. Autoimmune infiltration of islets grafted to the ear pinna is readily imaged directly through the skin allowing both dynamic and longitudinal imaging of lymphocyte responses. We observed that the T cells infiltrating the transplanted islets were less mobile than those swarming around in a naïve lymph node, most likely indicating that they had received activation through the TCR and were engaged with a target (57). This mirrors the behavior observed in tumor infiltrating T cells, which arrest on tumor cells expressing their cognate antigen (58, 59), the antigen-induced migration arrest of T cells infiltrating a mis-matched skin graft (60) and mycobacteria-specific T cells in a cognate antigen rich environment (61). When we administered the non-FcR binding anti-CD3 antibody, we saw a rapid increase in the mobility of the infiltrating T cells in the islets, more similar to the swarming behavior of T cells which have not yet found a suitable APC. A cessation of the TCR-MHC interaction, be it either CTL attacking a beta cell or a $CD4^+$ T cell receiving activating signals from an intra-islet APC, will naturally have dampening effects on the ongoing inflammation. As CTL function as "serial killers," moving from one target to the next (62), an interruption of the progress of just one cell can save several potential targets. Interaction time is also of crucial importance for acquisition of effector properties (43), and decreased interaction time between APC and T cell contributes to the tolerization of both $CD8^+$ T cells (33) and $CD4^+$ T cells (34), reducing their production of proinflammatory cytokines and expression of surface activation markers. The long-lasting effects of short-term anti-CD3 treatment involve the interruption and qualitative change of the anti-islet immune response, and non-specific bystander T cells are cleared from the islet just as the islet-specific effectors are. This indicates that anti-CD3 treatment affects not only the T cells, but by extension also the pro-inflammatory milieu in the islet, possibly *via* effects on antigen-presenting cells (9), breaking the cascade of proinflammatory events that would otherwise result in diabetes. Thus, the observed increase in motility may well influence both the short-term and long-term effects of anti-CD3 treatment, i.e., the cessation of interaction and downregulation of CD3, but also a skewing of the ensuing immune response to be more tolerogenic. In relation to the demonstrated need for TGFβ, we surmise that the consequent clearance of residual apoptotic cells during the resolution phase of healing may trigger release of active TGF-β, modifying the local microenvironment to acquire a level of immune privilege (63). In summary, imaging revealed a hitherto undescribed rapidity in response to treatment, with graft infiltrating cells recovering motility and thus terminating the cell–cell interactions required for T cell activation (47) within 20 min of injection of the aglycosyl anti-CD3 antibody. Repeated imaging of the same animal revealed the eventual loss of infiltrating T cells at the graft site.

Importantly, this technique offers an opportunity for reduction of the number of experimental animals used in research, as the non-invasive nature of the investigation allows longitudinal monitoring of the same islets in the same host. In addition, current models employing grafting of islets require a high degree of surgical proficiency while insertion into the ear pinna is minimally invasive. Traditional use of large groups of mice for temporal studies introduces experimental variation due to differing levels of immune infiltration in the pancreatic islet. The ability to collect data at several time points in the same experimental mouse negates this variance and enhances data quality. The amenable nature of beta islet grafting to the ear pinna makes this technique a new tool for studies of immune response in diabetes. The development of functional probes and new analytical tools is increasing the options for investigating immune responses *in vivo* with multiphoton imaging, and offers exciting opportunities for studies of type 1 diabetes. Combined use of IFN-γ reporter mice and Foxp3 reporter mice could clarify any differences in behavior of different cell subsets in the islets in response to treatment, and recent advances may soon allow studies of molecular dynamics in individual islet infiltrating cells (64). We have used islets transplanted into the pinna to clarify the events immediately following anti-CD3 treatment, and we now look forward to investigating the parameters required for establishment and maintenance of long-term tolerance. The use of the pinna as a site for islet transplantation could also facilitate studies of changes in islet mass over time and beta cell physiology (65, 66), and we hope that a combination of advances in both beta cell and immune cell imaging will lead to new insights into the biology of type 1 diabetes and thus, opportunities for treatment.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Animals (Scientific Procedures) Act. The protocols were approved by the UK Home Office and performed under the Project Licences of PG, KO, MC, and MW.

AUTHOR CONTRIBUTIONS

MW, RB, AR, JF, and FG performed the experiments. RB, MW, PG, FG, KO, JF, MC, and AC planned the experiments.

MW wrote the manuscript, RB, JB, PG, HW, AR, FG, KO, JF, MC, and AC contributed to discussion and reviewed/edited manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01006/full#supplementary-material.

MOVIE S1 | Kaede-C57BL/6 fluorescent islets transplanted into the pinna of the ear of a wt C57BL/6 mouse. Image capture starts at the bottom of the Z stack (middle of the ear) and moves to the top (up through the dermis to the epidermis). The movie shows the Kaede transplanted islets in green, vasculature in red (Qdot tracer given i.v. prior to imaging), and the blue is second harmonic autofluorescence generated by the dermal collagen fibers. Each stack is roughly 150 μm thick. Loops of blood vessels going around hair follicles (which are the round holes in the collagen network) show up in red.

MOVIE S2 | 16-min capture of pinna with NOD scid islets transplanted into a NOD-hCD2-GFP recipient. T cells are green (GFP), blood vessels are red (Q-dots), and collagen is blue (secondary harmonic signal).

MOVIE S3 | 24-min capture of pinna with NOD scid islets transplanted into a NOD-hCD2-GFP recipient. T cells are green (GFP), blood vessels are white (Q-dots), and collagen is blue (secondary harmonic signal), while dead cells become red through uptake of Sytox dye.

MOVIE S4 | 13-min capture of pinna with NOD scid islets transplanted into a NOD-Foxp3-GFP recipient. Foxp3+ T cells are green (GFP), blood vessels are red (Q-dots), and collagen is blue (secondary harmonic signal).

MOVIE S5 | 15-min capture of pinna with NOD scid islets transplanted into a NOD-hCD2-GFP recipient (depicted in Figure S1 in Supplementary Material) before injection of antibody. T cells are green (GFP), blood vessels are red (Q-dots), and collagen is blue (secondary harmonic signal).

MOVIE S6 | 15-min capture of pinna with NOD scid islets transplanted into a NOD-hCD2-GFP recipient (depicted in Figure S1 in Supplementary Material) after injection of isotype control antibody. T cells are green (GFP), blood vessels are red (Q-dots), and collagen is blue (secondary harmonic signal).

MOVIE S7 | 29-min capture of pinna with NOD scid islets transplanted into a NOD-hCD2-GFP recipient (depicted in Figure S1 in Supplementary Material) after injection of agly anti-CD3 antibody. T cells are green (GFP), blood vessels are red (Q-dots), and collagen is blue (secondary harmonic signal).

MOVIE S8 | 29-min capture of pinna with NOD scid islets transplanted into a NOD-hCD2-GFP recipient before injection of agly anti-CD3 antibody. T cells are green (GFP), blood vessels are red (Q-dots), and collagen is blue (secondary harmonic signal).

MOVIE S9 | 32-min capture of pinna with NOD scid islets transplanted into a NOD-hCD2-GFP recipient after injection of agly anti-CD3 antibody. T cells are green (GFP), blood vessels are red (Q-dots), and collagen is blue (secondary harmonic signal).

MOVIE S10 | 59-min capture of a pancreatic islet with immune cell infiltrate in a NOD-hCD2-GFP mouse before injection of antibody. T cells are green (GFP), blood vessels are red (Q-dots), and collagen is blue (secondary harmonic signal).

MOVIE S11 | 59-min capture of a pancreatic islet with immune cell infiltrate in a NOD-hCD2-GFP mouse after injection of agly anti-CD3 antibody. T cells are green (GFP), blood vessels are red (Q-dots), and collagen is blue (secondary harmonic signal).

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Tolerance Induction in Relation to the Eye

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Inflammatory intraocular eye diseases, grouped under the term uveitis are blinding conditions, believed to be mediated by pathogenic autoimmune processes that overcome the protective mechanisms of the immune privilege status of the eye. An animal model for these diseases, named experimental autoimmune uveitis (EAU), is induced by initiation of immunity against ocular-specific antigens, or it develops spontaneously in mice with T-cells that transgenically express TCR specific to the target eye antigen(s). T-Cells specific to ocular antigens are generated in the thymus and their majority are eliminated by exposure to their target antigen expressed in this organ. T-cells that escape this negative selection acquire pathogenicity by their activation with the target antigen. In spontaneous EAU, the microbiota play crucial roles in the acquisition of pathogenicity by providing both antigenic stimulation, by molecules that mimic the target ocular antigen, and an additional stimulation that allows invasion of tissues that harbor the target antigen. The pathogenic process is physiologically inhibited by the peripheral tolerance, composed of antigen-specific T-regulatory (Treg) lymphocytes. Deleting the Tregs enhances the ocular inflammation, whereas adoptively transferring them suppresses the pathogenic response. Potential usage of Treg cells for suppression of autoimmune diseases in humans is under intensive investigation.

Keywords: experimental autoimmune uveitis (EAU), ocular inflammation (uveitis), tolerance process, T-helper (Th) cells, T-regulatory cells (Treg), microbiota

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INTRODUCTION

The clarity of ocular tissues and spaces is crucial for sight. Inflammatory processes thus have detrimental effects on vision, by causing media opacity. Furthermore, the damaging effects of inflammation in the eye are also harmful by affecting photoreceptor cells that are critical for the process of vision, and do not regenerate. To protect against immune-mediated inflammatory processes, the eye has evolved multiple layers of defense that endow it with a status of immune privileged organ (1). These layers of defense, studied, and defined by many investigators over the course of recent four decades include: (i) an efficient blood-retina barrier; (ii) an immunoinhibitory environment composed of several soluble and cell-bound immunosuppressive molecules; (iii) an active process known as "anterior chamber associated immune deviation (ACAID)" which can be demonstrated experimentally, in which foreign antigens reaching the anterior chamber of the eye initiate a complex immunosuppressive process (2, 3).

Early on, it was thought that due to their sequestration in the eye, ocular antigens, in particular those of the retina, are treated as foreign and provoke immune responses when exposed to the immune system. This notion was supported by the features of Sympathetic Ophthalmia, a disease

in which trauma to one eye is followed by inflammation in the other, undamaged, eye. Autoimmunity has been proposed to play a major role in the pathogenesis of sympathetic ophthalmia, as well as in the pathogenesis of other human eye diseases in which intraocular inflammation without an accompanying infection, is the major symptom. These include birdshot chorioretinopathy, Behcet's disease, Sarcoidosis, and Vogt-Koyanagi-Harada (VKH) disease; other inflammatory eye diseases listed under the "uveitis" umbrella are not covered here.

Pathogenic autoimmunity is also assumed to be the mechanism responsible for phacoanaphylactic endophthalmitis, a rare but severe eye disease that follows damage to the lens and the release of lens antigens, proteins that are normally sequestered from the immune system (4).

As will be discussed below, the "physiological" approach to treating autoimmune-mediated diseases would be by induction of specific tolerance to the autoantigens that are the targets for the pathogenic response. We summarize here basic data concerning these issues, specifically dealing with tolerance to antigenic components of intraocular tissues of the eye. Immunity and tolerance to the cornea and external eye tissues have been investigated thoroughly and were reviewed elsewhere (5–7).

UVEITIS—CURRENT STATUS

Intraocular inflammatory conditions in humans are grouped under the term noninfectious uveitis, that includes the five eye diseases mentioned above (8, 9). Autoimmune processes are believed to play a major role in the pathogenesis of these diseases, a notion supported by findings of cellular and/or humoral immune responses against ocular auto-antigens in these patients (8–11). In addition, involvement of autoimmune processes is strongly supported by the similarity between the pathological changes specific to these human diseases and the ocular changes seen in animals with experimental autoimmune uveoretinitis (EAU) (8, 9, 12). This animal disease is induced by immunization with retina-specific antigens and is mediated by pathogenic T- lymphocytes (9, 13, 14). The notion that the human conditions are mediated by immunopathogenic T-cells is also supported by the finding that, like animal models (15, 16), patients with these conditions tend to respond positively to treatment with immunosuppressive agents that mainly target T cells, such as cyclosporine, rapamycin, and FK-506 (16–18) and, more recently, by daclizumab (antibody to the interleukin 2 receptor) and other biologics (19–21).

Treatment with immunosuppressive agents, however, inevitably has adverse effects on host defense. Therefore, the ideal treatment would be induction of antigen-specific tolerance. Selective tolerance to uveitogenic antigens has been achieved in animals, in which treatment with a uveitogenic protein (S-antigen/retinal arrestin) yielded inhibition of EAU induced by immunization with this same molecule (22, 23). In these studies the antigen was administered by oral gavage during the development of the experimental disease and Dick et al. (24) reported high efficiency of EAU suppression by intranasal administration of the retinal antigen. Since patients with uveitis often demonstrate immunity toward retinal S-antigen (10, 25), studies were carried out to examine the efficacy of treating such patients for induction of oral tolerance by feeding with this antigen (26), or with an HLA peptide that crossreacts with S-antigen (27). The initial results appeared encouraging, but additional studies are required to examine the therapeutic usefulness of the procedure in more depth. The main advantage of oral tolerance is the absence of known adverse effects, but its drawback, as with other antigen-specific tolerance approaches, is that it requires knowledge of the inciting antigens. However, the specific antigens that serve as molecular targets for uveitis are not certain. That said, the phenomenon of tissue specific bystander suppression, where regulatory cells induced to one antigen may suppress responses to other antigens in the same tissue environment (28) could, in theory, be exploited to get around this limitation.

CENTRAL AND PERIPHERAL TOLERANCE IN RELATION TO THE EYE

Over the past few decades, research with animals, primarily mice, has provided a large amount of information concerning the complex process of tolerance. The state of tolerance to self antigens is achieved by two separate processes: central and peripheral tolerance, which operate in tandem. When either of these fails, autoimmunity can result. Central tolerance occurs in the thymus. As part of their development, new T-lymphocytes with specificity toward a wide range of antigens are generated in the thymic cortex, and follow a stereotypic process of maturation through several well defined stages (29, 30). As part of this process, maturing T-cells with high affinity toward autoantigens undergo apoptotic death in the thymic medulla upon exposure to their cognate tissue antigens, whereas T cells with intermediate affinity may be converted into natural T regulatory cells (nTregs). The tissue antigens that trigger this process of thymic education were shown to be expressed ectopically by thymic medullary epithelial cells (29, 30).

Several groups have examined the process of central and peripheral tolerance toward retinal antigens, using transgenic mice expressing a neo-self antigen in the retina (hen egg lysozyme = HEL, or β-galactosidase = β-Gal) and/or a transgenic TCR to the neo- or a native self-antigen (31–36). With one exception, which may have been due to technical limitations (32), the conclusion reached by these studies is that there was detectable elimination of retina-specific T cells in the thymus. This was subsequently shown to be due to expression of retinal antigens (or retinal neo-antigens) in the thymus under control of the AutoImmune REgulator (AIRE) transcription factor that drives expression of tissue-specific antigens, including retinal antigens, in the thymus, and mediates negative selection of autoreactive lymphocytes (37, 38). Expression in the thymus of a natural uveitogenic retinal antigen, interphotoreceptor retinoid-binding protein (IRBP), was demonstrated by using RT-PCR (39) and immunohistochemistry (36) and was positively correlated in different mouse strains with their resistance to EAU (39) and with elimination of reactivity to specific uveitogenic epitopes from the

immune repertoire (40). Importantly, testing thymic expression of ocular-specific genes (S-antigen, IRBP, RPE65, and recoverin) in thymi of several human individuals revealed a remarkable variability in the level of expression of these molecules (41). These data thus provide a possible mechanistic explanation for the differences among individuals in their susceptibility to autoimmune uveitis and to tissue specific autoimmune diseases in general, suggesting that the susceptibility is regulated at least in part by the level of thymic expression of the pathogenic autoantigens.

It is also of note that AIRE-controlled expression of self antigens plays an important role in the generation of thymus-derived natural Treg cells (nTregs) (42, 43). This likely includes also IRBP-specific nTreg cells that might account for raising the threshold of susceptibility to EAU, even though after a uveitogenic challenge, disease may be regulated also by induced Tregs (43), elicited in the periphery as a result of the immunization and disease process.

The process of thymic central tolerance alone is insufficient to eliminate all the T-lymphocytes reactive to tissue-specific antigens and consequently, a proportion of self-reactive cells manage to exit to the periphery. Such escapee cells are normally kept in check by a process known as peripheral tolerance, namely, exposure to the cognate antigen in the tissue in the absence of costimulatory danger signals, precipitates immunological paralysis (anergy), or conversion to Tregs (dubbed peripheral or induced Tregs). In a curious way, this parallels the process of central tolerance, in that it requires contact with the cognate antigen, whereupon the autoreactive cells are disarmed.

In the case of the eye, however, peripheral tolerance may not operate efficiently due to limited accessibility of the tissue antigens which are unique to the eye, and are largely sequestered behind a blood retinal barrier. In support of this notion, retinal neo-antigen presentation is not detectable in eye-draining lymph nodes, and circulating TCR Tg lymphocytes specific to retina display a largely naïve phenotype (31, 33, 44, 45). Therefore, we believe that T cells that have not been deleted in the thymus persist in the periphery in a non-tolerant state. This notion is further supported by data showing that gene expression of retinal antigens outside the eye, e.g., by transgenesis, retroviral transduction, or hydrodynamic injection, confers profound resistance to EAU (33, 43, 46). Furthermore, healthy humans have a relatively high frequency of circulating T cells specific to retinal antigens (47). These concepts are illustrated in **Figure 1**. In the aggregate, the data paint a picture whereby tolerance to retinal antigens is dependent mainly on thymic selection, whereas peripheral tolerance mechanisms are a "weak link" that may present an opportunity of therapeutic manipulation.

NATURAL AND EXPERIMENTAL TRIGGERS OF UVEITIS

Despite their presence in the circulation, naïve autoreactive T cells clearly do not cause uveitis in most individuals; perhaps, because they are unable to invade the target organs without additional activation processes. It has been well established in models of autoimmune disease that in order to elicit disease by infusion of autoimmune lymphocytes, these cells must first be activated *in vitro*. The notion that circulating naïve T cells are incapable of invading normal tissues, is supported by findings in mice. Thus, naïve CD4 T cells specific to a neo-self eye antigen do not induce ocular inflammation, unless they are pre-activated (*in vitro)* (49) and the same was true of T cells that express transgenic TCR specific for the natural retinal antigen IRBP (44). This leads to the question, where do retina-specific uveitogenic lymphocytes become activated *in vivo* to acquire the ability to penetrate the blood-retinal barrier and infiltrate the eye. Importantly, exposure *in vivo* to innate stimuli, such as TLR ligands, or complete Freund's adjuvant (which contains heat killed mycobacteria), without *in vitro* preactivation of the autoreactive cells, could be sufficient to support induction of uveitis in both uveitis models mentioned above (39, 40). While additional effects on the host, such as increased leakiness of the vasculature (50), could not be excluded as contributing factors, these observations strongly suggest that microbial stimuli might be involved in triggering uveitis.

Extrapolating from the knowledge that innate microbial signals (e.g., complete Freund's adjuvant) drive tissue specific T cells to a pathogenic effector phenotype, Caspi and coworkers further explored the role of commensal microbiota as a trigger of uveitis. Toward that end, they developed the R161H mouse strain, which expresses an IRBP specific TCR and develop autoimmune uveitis spontaneously (44). Rearing the mice under cover of broad spectrum antibiotics, or under germ-free conditions, strongly attenuated development of disease, supporting the notion that commensal flora can serve as a trigger of autoimmune uveitis (45). Importantly, in microbe-containing mice, the retina-specific R161H cells were seen to signal through their IRBP-specific receptors in the intestine, suggesting that they were being activated *in situ* by commensal flora in an antigen specific fashion to trigger disease. The proposed scenario of the gut-eye axis in uveitis is depicted in **Figure 2**. Interestingly, Gery and colleagues found (51, 52), that the process of pathogenicity acquisition by uveitogenic T-cells requires an additional phase of 2–3 days in the spleen, and possibly other organs, during which *in vitro* activated autoimmune lymphocytes are "licensed" to invade tissues where the target antigen is located. Similar findings were reported for the brain, another immune-privileged tissue (53).

IMMUNE PRIVILEGE AND UVEITIS

As briefly discussed above, the eye is protected from the immune system by the complex phenomenon of immune privilege, in which sequestration is only the first layer of defense. The internal environment of the eye contains a variety of potent soluble and cell-bound inhibitory molecules, including TGF-β, α-MSH, CGRP, VIP, and retinoic acid, as well as FasL, PD-L1, TSP-1, to name a few [reviewed in ref (3)]. Multiple studies over the last 3 decades presented evidence that ocular fluids and ocular resident cells inhibit activation and function of various immune

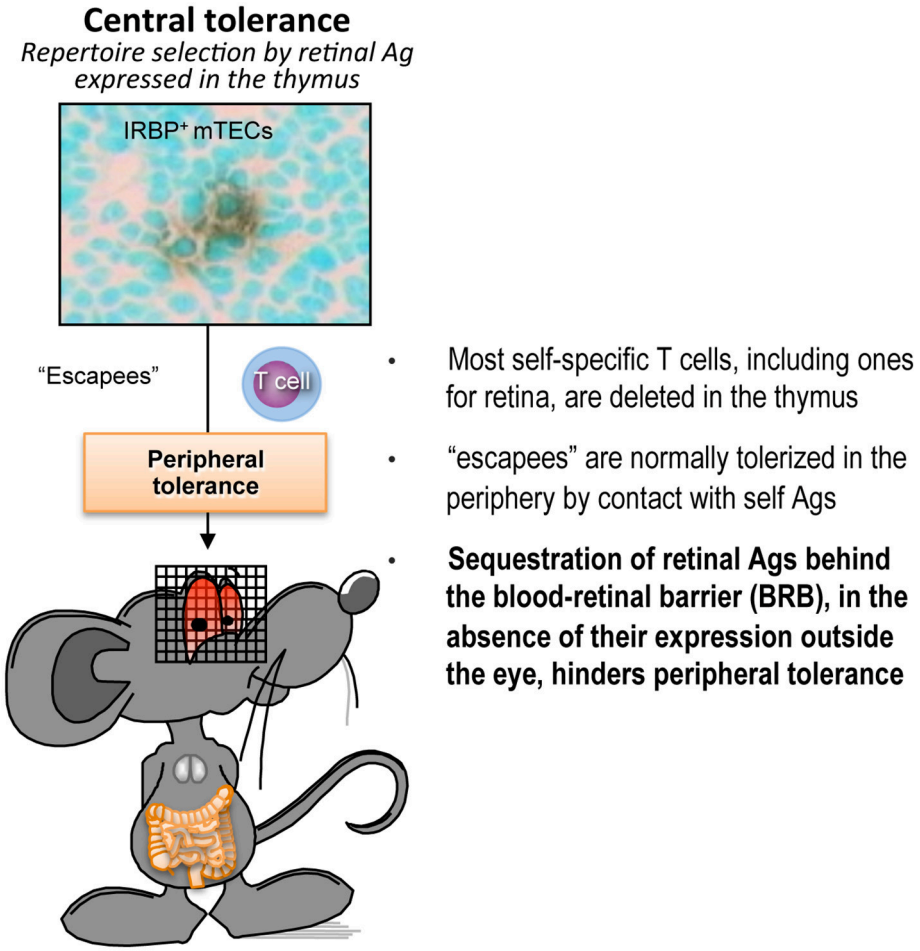

FIGURE 1 | Peripheral tolerance is inefficient in the case of retina-specific T cells. Schematic representation of the process of self-tolerance to retinal antigens. Adapted from Horai and Caspi (48). No copyright permissions are required for the reuse of this image.

cells in culture, and can even convert T helper (Th) cells to Foxp3$^+$ Tregs (54). How, then, can we explain induction of uveitis in the face of immune privilege? To examine whether the living eye has the ability to control activation of uveitogenic T cells, Caspi and colleagues injected IRBP-specific T cells obtained from R161H donor mice directly into eyes of recipient mice, and followed their fate. The data showed that naïve R161H T cells recognized their cognate antigen but did not acquire effector function in the eye. Instead, they converted to Foxp3$^+$ Treg cells, which were functional and were able to inhibit activation of fresh R161H T cells in a standard T cell suppression assay (54). In contrast, antigen-experienced R161H T cells, isolated from IRBP immunized R161H mice by selection for activation/memory markers, that had been exposed to IRBP before being placed in the eye, could not be inhibited by the suppressive intraocular environment and caused extensive photoreceptor damage (54). This can explain why T cells that have been activated outside the eye—whether as a result of trauma or activation in the gut—and have acquired the ability to penetrate the blood-retinal barrier, can induce uveitis despite ocular immune privilege.

NATURAL AND THERAPEUTIC REGULATION OF UVEITIS

Peripheral tolerance against pathogenic autoimmunity is executed by several types of regulatory cells, including, but are not limited to, natural, and induced Tregs. Treg cells are capable of killing and/or suppressing T-lymphocytes specific to autoantigens, that escaped the negative selection process in the thymus and migrated into the blood and lymphoid organs [reviewed in ref (55)]. In early studies, the CD4 Treg cells were identified mainly by their strong surface expression of CD25 (56), but their identification has been greatly improved by the finding that their majority express the transcription factor FoxP3. Depletion of Foxp3+ Tregs caused the mice to develop more severe uveitis upon IRBP challenge, indicating that preexisting (likely thymic-derived) Tregs raise the threshold of susceptibility to uveitis (57). In mice that already developed uveitis, depletion of Foxp3+ Tregs at the peak of disease prevented resolution, and depletion after resolution induced a relapse, indicating that Foxp3+ Tregs naturally bring about and maintain resolution

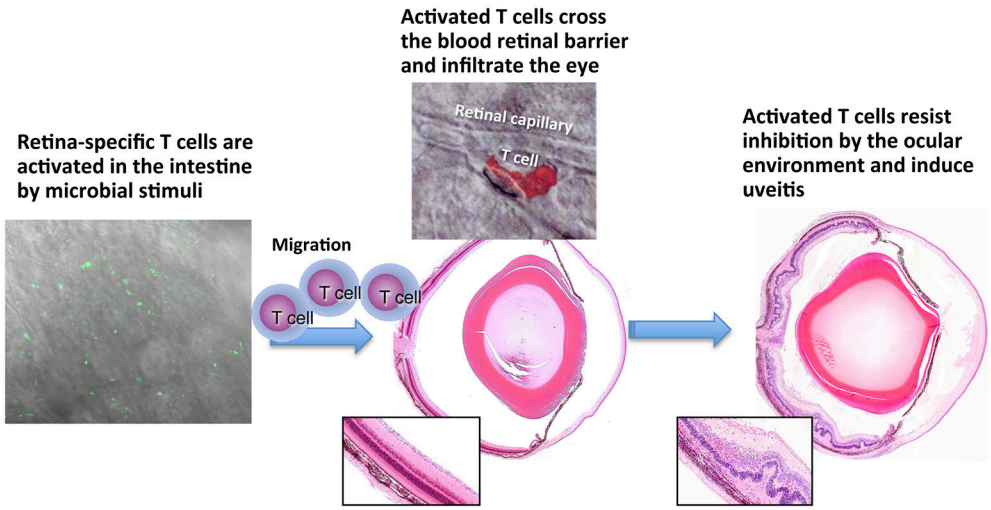

FIGURE 2 | Persisting retina-specific T cells can be activated by peripheral stimuli**.** Schematic representation of EAU induction after activation of retina-specific T cells in the gut. Green cells in left panel are nurIlgfP-positive T cells that are signaling through their T cell receptor [Horai et al. (45)]. Red cell in the middle top panel is a retina-specific T cell extravasating from a retinal vessel. The cell is stained by the PKH26 tracking dye and this photo was previously published in Prendergast et al. (50). No copyright permissions are required for the reuse of this image.

of EAU (43). The suppressive activity of Treg cells in the EAU system was also examined by Gregerson group, who found that Tregs may be generated in the eye and protect the retina from EAU induced by active immunization or adoptively transferred pathogenic Th cells. Interestingly, the generation of local Treg cells is inhibited by selective elimination of dendritic cells (DC) and microglia cannot replace DC in this function (58).

Treg cells that do not express Foxp3 (Tr1) have also been reported and characterized (59). Tr1 cells may be important in uveitis, but less is known about them, as they are more difficult to identify, and therefore to study. Tr1 cells are induced by IL-27 (60), as well as by c-Maf, Il-21 and ICOS (61). Caspi group demonstrated that IL-27 is potently induced in DC by IFN-γ-producing natural killer (NK) cells, in a self-amplifying feedback loop that takes place in the draining lymph node of mice immunized for uveitis. This interaction dampens the immune response that leads to uveitis, thus identifying NK cells as a novel regulatory cell that controls the magnitude of the autoimmune response (62). Other cells identified to be immunosuppressive in the context of EAU are IL-35 producing B-cells that were recently found by Egwuagu and colleagues to suppress EAU development, in part through induction of IL-35-producing Tregs (63, 64). Interestingly, this group showed that the IL-12p35 subunit has immunoregulatory functions that were hitherto attributed to IL-35 (65). IL-35 is also expressed by Treg cells and, interestingly, Wei et al. reported that that different subpopulations of Tregs produce IL-35 or IL-10 (66).

Based on the data described above, the group of Caspi set out to examine whether therapeutic induction of tolerance could regulate the pathogenic process of EAU. Treatment of mice with an IRBP expression plasmid, in the form of naked DNA administered by hydrodynamic injection, markedly suppressed the induction of EAU in the treated mice and analysis of the inhibitory process revealed that the inhibition of EAU was mostly due to CD4+CD25+FoxP3+ regulatory cells (43). Notably, Treg cells from hydrodynamically injected mice could be expanded into functionally suppressive Treg cell lines that, when adoptively transferred to mice immunized with IRBP, inhibited EAU development in the recipient mice (43).

In view of the specific and potent inhibitory capacity of Treg cells, the notion of using these cells in suppression of autoimmune disease in humans seems very attractive (67). One approach is to isolate antigen-specific Treg cells, increase their numbers *ex vivo* and inject them back to the patient (68). Another approach, proposed by the Salomon group (69), offers uveitis as the model disease, and uses preactivated polyclonal Treg cells that would exert bystander suppression in the target tissue. The system was tested in mice with EAU and suppression of disease was achieved, but only when the cells were injected into the vitreous. So far, no data have been reported to show successful treatment of uveitis patients with Treg cells. However, the notion that Treg cells are involved in suppression of the pathogenic process of uveitis is supported by the finding of a correlation between increase in the proportions of Treg cells in blood of uveitic patients and remission of the disease (70). Furthermore, the frequency of Treg cells was found to decline in parallel with increase in the severity of the uveitic changes (71).

In addition to T and B cells, myeloid cells can also regulate EAU. Myeloid-derived suppressor cells (MDSC) are a rather heterogeneous myeloid cell population that may include monocyte/macrophage- and granulocyte-like populations. Evidence reported by several groups indicates that MDSC could act at the systemic as well as local levels to curb disease (72, 73).

Importantly, MDSC have also been identified in association with human uveitis (73). Additionally, dendritic cells (DC) having a tolerogenic phenotype may also play a regulatory role. Forrester and collaborators reported that treatment of mice with LPS-induced tolerogenic DCs, which produced IL-2 and suppressed uveitis by multiple mechanisms (74).

Finally, ocular inflammation may also be regulated by non-lymphoid cells, known as mesenchymal stem/stromal cells (MSCs). The mechanisms may involve induction of MDSC-like cells (75) as well as induction of Treg cells (76).

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

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MINI REVIEW
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Heat Shock Proteins Can Be Surrogate Autoantigens for Induction of Antigen Specific Therapeutic Tolerance in Rheumatoid Arthritis

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Technologies that enable induction of therapeutic tolerance may revolutionize the treatment of autoimmune diseases by their supposed potential to induce drug-free and lasting disease remission. In combination with diagnostic tests that screen for individuals at risk, these approaches may offer chances to halt disease before serious damage in the tissues can occur. In fact, for healthy individuals at risk, this could lead to a preventive form of vaccination. For therapeutic tolerance to re-instate natural self-tolerance it seems essential to induce tolerance for the critical autoantigens involved in disease. However, for most autoimmune diseases such antigens are poorly defined. This is the case for both disease inciting autoantigens and antigens that become involved through epitope spreading. A possible source of surrogate auto-antigens expressed in tissues during inflammation are heat shock proteins (HSP) or stress proteins. In this mini-review we discuss unique characteristics of HSP which provide them with the capacity to inhibit inflammatory processes. Various studies have shown that epitopes of HSP60 and HSP70 molecules can function as vaccines to downregulate a variety of autoimmune inflammatory diseases. Currently, several research groups are developing cell therapies with the intention to reach therapeutic tolerance. In this review, in which we are proposing to *ex vivo* load tolerant dendritic cells with a Treg inducing HSP70 derived peptide called B29, we are discussing the chances to develop this as an autologous tolDC therapeutic tolerance therapy for rheumatoid arthritis.

Keywords: stress protein, heat shock protein, rheumatoid arthritis, tolDC, autoimmunity

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INTRODUCTION

Although originally discovered by their enhanced expression following the rise of temperature, the enhanced expression of HSP is now known to depend on a multitude of factors which include the mediators associated with inflammation. Research directed toward the role of HSPs in inflammatory diseases has put emphasis on the involvement of the HSP60, HSP70, and HSP90 molecules. In the new nomenclature these molecules are known as HSPD, HSPA, and HSPC (1). These families of larger HSPs tend to display strong evolutionary conservation from bacteria to humans (2). Recognizing the role of immune encounters with microbiota, such as in the tolererizing gut mucosal tissues, suggests that due to this evolutionary conservation T cells adopt a tolerizing

phenotype upon their encounter with these abundant and omnipresent molecules. The larger family of HSP molecules is the HSP70 or HSPA family. Some HSP70 proteins are constitutively expressed, while some others are highly stress inducible.

EXPRESSION OF HEAT SHOCK PROTEINS IN STRESSED CELLS AND INFLAMED TISSUES

Peptide elution profiles obtained from MHC class II molecules have revealed the frequent presence of HSP70 derived peptides in MHC-II. This was in particular the case for MHC-II of stressed cells, possibly resulting from the known role of HSP70 proteins in the process of chaperone mediated autophagy. Paludan et al. (3) have analyzed the proteomes of both MHC-I and MHC-II molecules during virus infection, both mouse and human. In this study the three most frequent nuclear/cytosolic natural ligand sources that were defined for MHC-II were HSC70, HSP70, and GAPDH. Another study by Dengjel et al. (4) analyzed the proteome of an HLA-DR4 molecule obtained from human B cells stressed by nutrient deprivation. Also in this study the dominant presence of HSP70 fragments in this proteome was said to result from autophagy. Interestingly enough, one of the HSP70 peptides that was eluted appeared to be identical with a peptide, called mB29b, representing an epitope recognized by regulatory T cells earlier defined by us in our experimental arthritis model (5). A report dealing with the enhanced expression of HSP in tissues during inflammation can be found in Schett et al. (6). In this study expression of HSP70 was shown for the synovial tissue of RA joints. In addition the effect of fever and TNFα driving the cellular expression of HSP70 and its transcription factors was shown.

TOLERANCE DRIVEN BY MICROBIAL—MAMMALIAN HSP T CELL CROSS-RECOGNITION

The first evidence for tolerizing effects of HSP was obtained in the model of heat-killed mycobacteria induced arthritis in Lewis rats, the model of adjuvant arthritis (7). We cloned HSP60 from mycobacteria and showed that this antigen was the main T cell target in the model. However, HSP60 immunization did not produce any arthritis and such immunized animals were shown to become resistant to induction of adjuvant arthritis. Interestingly, HSP60 immunization also was shown to protect in other models, such as arthritis induced with streptococcal cell walls (8) and arthritis induced with an oily compound called pristine (9). Besides arthritis, also models of diabetes and atherosclerosis showed the protective effects of mycobacterial HSP60 [reviewed in van Eden et al. (10)]. By analyzing T cell epitopes of mycobacterial HSP60 in Lewis rats we detected the 256–265 sequence being very conserved. T cells specific for 256–265 cross-responded to the mammalian HSP60 molecule and were found to protect against disease upon adoptive transfer in the adjuvant arthritis model (11). T cells raised against non-conserved epitopes did not protect. Similar results were obtained more recently for HSP70 in a model of proteoglycan induced arthritis in mice. In this model, the B29 peptides, of which mB29b was already mentioned here above to be present in the MHC-II elution profile of stressed cells, were found capable of inducing protective T cells based on microbial-mammalian cross-recognition (5).

Before introducing the possible mechanisms leading to the tolerance promoting activities of HSP peptides we describe in more detail our B29 peptide. This peptide of mycobacterial origin is 15 amino- acids long with the following sequence: VLRIVNEPTAAALAY. Its mammalian homolog mB29b: VLRIINEPTAAAIAY. Both peptides were high to moderate MHC binders, when tested in competitive binding assays, for most human HLA class II molecules, such as HLA-DR1, DR3, DR4, DR11, DQ2, and DQ8 (12). Their functionality and protective effects in the context of human HLA molecules were shown in a model of proteoglycan induced arthritis (PGIA) carried out in a HLA-DQ8 transgenic mouse (12). Also T cell responses in humans with specificity for these peptides, including cross-reactivities between B29 and its mammalian homologs, were documented (12).

How the microbiota impacts the immune system has been analyzed in great detail over recent years. For instance, mucosal dendritic cells (DC) are now known to regularly sample the microbiota and to present their antigens to T cells (13). It may be inferred that the bacterial stress response that evolves following ingestion leads to upregulation of microbial HSP and that therefore microbial HSP peptides are frequently seen by the mucosally residing Tregs. Along similar lines it can be understood that the more conserved peptide sequences will dominate, given their more frequent and repetitive presence in the antigenic make-up of our diverse microbiota. In this manner it is possible that the basis for induction of Treg responses by conserved HSP peptides is imprinted into the immune system at the mucosa or other sites of the body where contact with microbiota occurs. Therefore, it is possible that a combination of stress inducibility and the evolutionary conservation has provided HSP with the capacity to control inflammation.

HSP DAMPEN IMMUNE RESPONSES

Since the molecular cloning of HSP60 from mycobacteria, many studies were done with recombinant, in *E. coli* produced, HSP proteins. Insufficient attempts to clear these proteins from contaminating LPS has resulted in false positive observations and reports of immune activation. However, several subsequent studies have shown that pure HSP preparations were lacking immune stimulating activities (14). Nonetheless, in too many cases HSP are listed as example molecules with damage associated molecular patterns or DAMPs. Apart from the fact that clean molecules were shown to lack DAMP qualities, many arguments exist that further disqualify HSP for being DAMPs (15). By their nature DAMPs are exclusively intracellular and are supposedly only released by cells upon damage. HSP, however, are known to be present in body fluids, such as for example serum. Furthermore, it was shown in

various studies that HSP can have immuno-modulatory effects on DC. In the mouse PGIA model, HSP70 treated DCs loaded with proteoglycan, were found, upon *in vivo* transfer, to suppress disease. In addition, HSP70 treated DC loaded with OVA, were found to induce production of IL-10 in OVA specific T cells (16). These findings were made with both mycobacterial and murine HSP70. In a different set of experiments carried out by others, mycobacterial HSP70 was shown to impair the maturation of bone marrow derived DC, to induce IL-10 production and to inhibit T cell proliferation (17). These findings, together with the reported disease inhibitory activities of HSP molecules, are pleading against HSP being DAMPs, but indicative of their immune DAMPing capacities instead.

RHEUMATOID ARTHRITIS AS A MODEL AUTOIMMUNE DISEASE

Despite recent advances in the treatment of RA using a range of biological therapies (for example anti-TNF, CTLA-4, and anti-B-cell therapies), very few patients achieve long-term clinical remission, even when therapies are started early. A major challenge for research and drug development is now to find ways to change the outcome, with the aim of reaching sustained remission or cure in a large majority of patients. Patients that benefit from biological therapies are treated with weekly to monthly/half-yearly injections and continued use of oral methotrexate, with unpleasant side-effects, and sometimes serious and life-threatening adverse effects due to suppression of the immune system. The ultimate therapeutic ambition for rheumatologists is to provide drug-free remission for all patients. Theoretically this could be achieved using a short course or infrequent (vaccine-like) treatment to restore normal immunity and prevent further synovial damage to maintain joint function. Such a therapy would not only benefit patients with established RA, it could also be used before the onset of RA to halt the disease process at the early immune initiation phase of the disease, before any joint damage has occurred. A summary of clinical trials developed for HSP based interventions in RA is given in the **Table 1**.

The HSP10 study was lacking a placebo group, which impaired full interpretation of results. The most recent study with HSP70 showed significantly prolonged remissions at the highest protein concentrations administered (5 and 15 mg). This in itself has suggested the induction of Treg, supporting the original hypothesis regarding the therapeutic action of HSP.

Improved understanding of the role of regulatory T cells and dendritic cells in suppressing the immune response may also lead to novel therapeutics to induce immune tolerance. Immune tolerance could be achieved using a combination of existing therapies, novel drugs and cell-based therapies and peptide immunotherapy to re-regulate and suppress the pathogenic immune response in RA. RA is an ideal disease setting for the study of tolerance inducing therapies. This is due to recent insights into the immunology of RA, coupled with advances in autoantibody identification and T and B-cell monitoring.

A CELL THERAPY APPROACH IN COMBINATION WITH HSP FOR RHEUMATOID ARTHRITIS

To develop a lasting cure through tolerance therapy for RA is an attractive, but certainly great challenge for immunologists. Since the discovery of dendritic cells as central elements in the initiation of cellular immune responses, the idea has surfaced that DCs are critical in the organization of tolerance and that resting tissue DCs may well have a role in presenting autoantigens in default manner for inspection by Tregs. Be that as it may, antigen presentation by DCs in a suboptimal manner may lead to induced Tregs (iTreg) (22), and as a consequence such iTreg do further impose tolerogenicity in other DCs, amongst others through their production of immunoregulatory cytokines, such as IL-10 and TGF-β. In this manner a self-sustaining cycle of tolerance induction may become operationally active. At the same time, through linked recognition, these cytokines may act on other naïve T cells and recruit them into the pool of iTregs, a process known as induction of infectious tolerance (23, 24). Through such mechanisms a lasting and spreading tolerance for a multitude of self-antigens may be created. In theory this situation may be imposed on an immune system in an inflammatory state, by instilling artificially tolerized DC into the system. The rheumatology group of Newcastle University (UK) has pioneered such approach in a first clinical trial (25). Monocyte derived DCs, taken from patients with RA, were cultured in the presence of VitD3 and dexamethasone, loaded with synovial fluid collected from patients and injected back into their inflamed joints. In this autologous cell therapy, tolerization was sought for the mixture of substances in the synovial fluids taken from inflamed joints which supposedly included critical RA auto-antigens. With this trial the logistics for such an approach were developed and the procedure was proven safe. However, with a complex mixture of antigens such as in synovial fluid, monitoring the immunological effect was complicated. Moreover, the necessity of including the patients with more advanced disease, with severely inflamed joints, for obtaining synovial fluids, was not advantageous. In collaboration with the Newcastle group we are now planning a similar trial with autologous tolDC, but now with HSP70 peptide B29 loaded tolDC (**Figure 1**). This Phase I-II, unblinded, longitudinal study will include 22 RA patients who will be treated with either a low dose (5×10.6) or a high dose (15×10.6) of cells intracutaneously or intranodal. The patients will be in remission or with low disease activity under conventional therapy. Exact monitoring of the effect of this tolerance therapy will be possible by tracing and characterizing B29 specific T cells following our B29 tolDC intervention. In other words, besides monitoring of adverse events as the primary outcome, we will monitor peptide specific T cell responses.

CELL THERAPY AND BEYOND

For the clinical use of our novel proposed therapy, the specificity of the induced effects with antigen loaded tolDC, is more

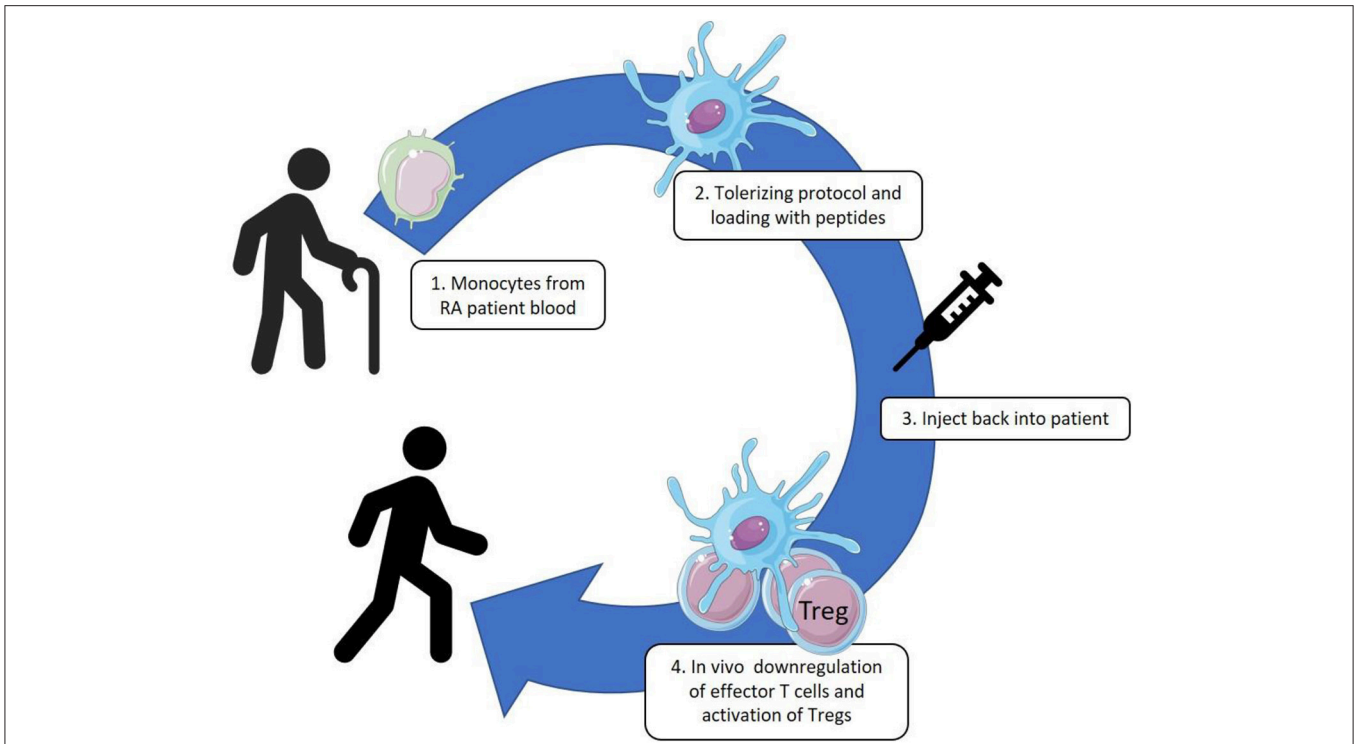

FIGURE 1 | Monocytes are collected from patient blood by leukapheresis and cultured with GM-CSF. These cultured DC-like cells are treated with VitD3-Dexamethasone, matured by monophosphoryl lipid A (MPLA) and loaded with the HSP70-B29 peptide. The tolerized and antigen loaded DC are injected back into the patients by intracutaneous or intranodal delivery.

TABLE 1 | HSP based clinical trials in inflammatory arthritis.

HSP product	Nr of patients	Administration	Effect	References
HSP40 (DnaJ) peptide: DnaJP1	RA $N = 15$	Oral 6 months	Increased IL-4 and IL-10. Decreased IL-2, IFN-γ, TNF and T cell proliferation	(18)
HSP40 (DnaJ) peptide: DnaJP1	RA $N = 160$	Oral 6 months	Clinical response, ACR20. Less T cells producing TNF	(19)
HSP10 (Chaperonin10)	RA $N = 23$	Intravenous 12 weeks (twice in a week)	Clinical improvement of disease activity	(20)
HSP70 (BiP)	RA $N = 24$	Intravenous (single administration)	Some patients with clinical and biological improvements	(21)

appealing and likely more safe with less general immunosuppression than we see with the currently available therapies. And moreover, already for the purpose of immune monitoring the antigen specificity of the intervention will be an essential and enabling aspect. An obvious possible limitation of the use of tolDC in chronic inflammatory disorders is that tolDC may not be able to utilize their tolerizing activities in the context of an ongoing inflammation. However, this potential limitation may possibly become negated by a synergistic effect of the intrinsic capacity of HSP to induce Tregs and of tolDC to convey immunological tolerance. As already mentioned by others, after biologics, the use of human cells as versatile therapeutic engines can be a new revolution in pharmaceutical practices (26). And especially for tolerance therapies the use of Tregs and tolDC may turn out to be effective approaches. Although costly, the prospect of lasting cure may well-compensate for high costs of tolerance therapies in chronic and severely debilitating disorders. For practical purposes however, the delivery of antigens directly *in vivo*, and directed to the relevant cells *in vivo* may be an attractive next step. To reach tolDC targeting *in vivo* it is for instance possible to exploit a novel tolerance-inducing technology that utilizes several components. Examples

are Poly Lactic-co-Glycolic Acid (PLGA)-Polyethyleenglycol (PEG)-PLGA hydrogels for sustained presentation of peptides, liposomal vitamin D3 for enhanced tolerization of targeted peripheral APC, or PLGA particles loaded with TNFα-siRNA. In earlier studies we have seen that nasal administration of B29 in PLGA particles was more effective in the prophylactic suppression of arthritis in the PGIA model than peptide alone (27). In addition, PLGA particles were found to induce a tolDC-like phenotype by enhancing retinaldehyde dehydrogenase (RALDH) in DC and to upregulate Foxp3 in T-cells (28). Incorporating vitamin D3 liposomes in such a slow release matrix (hydrogels) can enhance *in vivo* induction of tolDC comparable to current *in vitro* culture protocols to induce tolDC for cell therapy (29). Treatment of epidermal Langerhans cells with the active form of vitamin D3 generates functional $Foxp3^+$ Tregs through a mechanism dependent on keratinocyte-derived TGF-β. In contrast, treatment of dermal DCs with 1, 25-$(OH)_2$vitamin D3 generates functional IL-10^+FoxP3^- T_R1 cells in an IL-10-dependent fashion (30).

In combination with tolDC interventions it may be needed to interfere with the production of pro-inflammatory cytokines such as TNF. Short interfering RNAs (siRNA) hold a promising therapeutic potential against a variety of disease conditions. However, actual potency seems limited by its physicochemical properties i.e., high hydrophilicity and poly-anionic phosphate backbone leading to negligible cellular permeation and subsequent siRNA delivery. Recently, lipid-polymer hybrid nanoparticles (LPNs) have been shown to act as efficient carriers for intracellular delivery of siRNA both *in vitro* and *in vivo* (31).

CONCLUSION

Antigen specific tolerance therapies for autoimmune diseases are currently under development. In many cases however, the critical autoantigens are still escaping proper identification. Heat shock proteins are attractive molecules as surrogate auto-antigens and may have several advantages in comparison with regular autoantigens. Their conserved nature and their abundant presence in the MHC II peptidome of stressed cells in particular, seems to provide them with unique characteristics as targets for regulatory T cells. It is hoped that first clinical trials with HSP loaded tolDC, such as are now being developed for rheumatoid arthritis, will pave the way for further tolerance therapies, leading to medication free lasting remission of disease.

AUTHOR CONTRIBUTIONS

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

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