HLA-Restriction of Human Treg Cells Is Not Required for Therapeutic Efficacy of Low-Dose IL-2 in Humanized Mice

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Regulatory T (Treg) cells are essential to maintain immune homeostasis in the intestine and Treg cell dysfunction is associated with several inflammatory and autoimmune disorders including inflammatory bowel disease (IBD). Efforts using low-dose (LD) interleukin-2 (IL-2) to expand autologous Treg cells show therapeutic efficacy for several inflammatory conditions. Whether LD IL-2 is an effective strategy for treating patients with IBD is unknown. Recently, we demonstrated that LD IL-2 was protective against experimental colitis in immune humanized mice in which human CD4+ T cells were restricted to human leukocyte antigen (HLA). Whether HLA restriction is required for human Treg cells to ameliorate colitis following LD IL-2 therapy has not been demonstrated. Here, we show that treatment with LD IL-2 reduced 2,4,6-trinitrobenzensulfonic acid (TNBS) colitis severity in NOD.PrdcscidIl2rg−/− (NSG) mice reconstituted with human CD34+ hematopoietic stem cells. These data demonstrate the utility of standard immune humanized NSG mice as a pre-clinical model system to evaluate therapeutics targeting human Treg cells to treat IBD.

Keywords: IBD, IL-2, humanized, colitis, Tregs

INTRODUCTION

IBD is a chronic relapsing and remitting disorder broadly classified as either Crohn’s disease (CD) or ulcerative colitis (UC) (1–3). UC is an idiopathic disease of the large intestine with continuous lesions, whereas CD may occur anywhere along the gastrointestinal tract and is characterized by discontinuous lesions often with transmural inflammation. Over 1.4 million individuals are afflicted with IBD in the United States alone, carrying an annual economic burden of nearly $30 billion and an estimated 6–8 million patients world-wide (4–7). The pathogenesis of IBD is multifactorial and
driven by immunological, environmental, genetic, and microbial factors (8–10). Dysfunctional regulatory mechanisms of the immune system can trigger disease onset or exacerbate IBD. In recent years, genome-wide association studies have identified polymorphisms that confer increased risk for IBD with many occurring to genes involved in immune regulation or host defense (9, 10). Nevertheless, the molecular mechanisms driving IBD are not fully understood (10–13).

While there is no cure for IBD, current strategies to induce remission include immunosuppression or biologics that neutralize pro-inflammatory cytokines or impede immune cell trafficking (14–16). Though many patients initially respond to these therapeutic approaches, long-term immune suppression may increase the risk of infection and many patients that do respond become refractory to treatment. Recent efforts to selectively augment the number or function of T<sub>reg</sub> cells to treat chronic inflammatory diseases have yielded promising results (17). Work from Desreumaux and colleagues shows that following the transfer of ex vivo expanded autologous antigen-specific T<sub>reg</sub> cells is effective in treating refractory CD (18). However, access to good manufacturing practice-compliant facilities may limit broad implementation of such approaches. It has been appreciated for some time that T<sub>reg</sub> cells constitutively express CD25, a component of the trimeric IL-2 receptor complex, which increases the affinity of the receptor complex for IL-2 (19). This property enables T<sub>reg</sub> cells to bind IL-2 at lower concentrations compared to conventional T cells. Recent work has documented the efficacy of LD IL-2 in the treatment of various chronic inflammatory disorders including graft-versus-host disease, systemic lupus erythematosus, Wiskott-Aldrich syndrome, and hepatitis C virus-induced vasculitis due to the selective expansion of T<sub>reg</sub> cells (20–23).

Logistical and ethical considerations involving human subject research, combined with biological limitations of cell culture, make murine models attractive surrogates to study human diseases (24). However, investigations pertaining to immune regulation may limit their utility when evaluating host disease, systemic lupus erythematosus, Wiskott-Aldrich syndrome, and hepatitis C virus-induced vasculitis due to the selective expansion of T<sub>reg</sub> cells.

Histocompatibility complex (MHC) class I/II molecules. Previously we developed human immune system (HIS)-mice, adaptive immune responses are notoriously sub optimal due to thymic selection of human T cells being restricted to murine major histocompatibility complex (MHC) class I/II molecules. Previously we developed NSG mice deficient for MHCII and in which human CD4 T cells were selected and restricted to human leukocyte antigen (HLA). Using these mice, we established that HLA-restriction was required to phenocopy a human disease due to the patient’s monogenic immune disorder (26). We then employed HLA-restricted humanized mice as a pre-clinical model to assess the efficacy of LD IL-2 in IBD and found that LD IL-2 expanded human T<sub>reg</sub> cells and was protective against experimental colitis (28). Nevertheless, it is unclear if LD IL-2 T<sub>reg</sub> cell expansion and suppression of effector responses requires T<sub>reg</sub> cell T cell receptor-HLA interactions. Here, we report that the therapeutic effects of LD IL-2 is independent of T<sub>reg</sub> cell HLA-restriction and demonstrate the utility of immune humanized NSG mice as a platform to evaluate therapeutics targeting human T<sub>reg</sub> cells.

**METHODS**

**Humanized Mice**

NOD.Prkdc<sup>scid</sup>Il2rg<sup>–/–</sup>/SjIfm1Wjl/SzJ (NSG) mice reconstituted with human CD34<sup>+</sup> hematopoietic stem cells (HSCs) showing more than 25% human CD45<sup>+</sup> cells in peripheral blood were procured from The Jackson Laboratory (Bar Harbor, ME, USA). HSCs from four unique donors were used to reconstitute mice. All mice were maintained in the specific-pathogen free facility and animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Vanderbilt University Medical Center, Nashville, TN, USA.

**Immunophenotypic Characterization and Antibodies**

Immune cells were isolated from blood and spleens of NSG humanized mice at the experimental endpoint. Single cell suspensions of mouse blood and spleen underwent red blood cell lysis using ACK lysis buffer (ThermoFisher, Waltham, MA, USA) followed by passage through 70 µm cell strainer (BD Biosciences, San Jose, CA, USA). Immune cells were first blocked in 10% rat serum, followed by the incubation with fluorochrome-conjugated antibodies for 15 min at 4°C in PBS and washed with PBS containing 2% fetal calf serum (FACS buffer). Antibodies used for immunophenotyping: CD3, clone SK7 and CD8, clone RPA-T8 were purchased from ebioscience (San Diego, CA, USA). CD25, clone M-A251; CD45, clone HI30; CD127, clone A019D5; ICOS, clone C398.4A; CTLA4, clone BN13; CD45RO, clone UCHL1; CD69, clone FN50; HLA-DR, clone L243; CD45RA, clone HI100; CD16, clone 3G8 and FOXP3, clone 206D were purchased from BioLegend (San Diego, CA, USA). CD4, clone RPA-T4 antibody was purchased from BD Biosciences (San Jose, CA, USA). Live/Dead fixable aqua dead cell stain kit was purchased from Thermo Fisher. Intracellular FOXP3 staining was performed by employing the ebioscience™ FOXP3/Transcription Factor Staining Buffer Set according to the manufacturer’s protocol. The anti-FOXP3 monoclonal antibody (clone 206D) was purchased from ebioscience. Samples were acquired using a LSRFortessa flow cytometer (BD Biosciences) and data were analyzed using FlowJo Software (Tree Star, Inc., Ashland, OR, USA).

**Low-Dose IL-2 Treatment**

IL-2 (Proleukin™, Prometheus Laboratories Inc., San Diego, CA, USA) was reconstituted in PBS and mice were administered daily injections of 10,000 IU starting at the time of sensitization and continued until the experimental endpoint.
Induction of Colitis Using TNBS
Mice were anesthetized using 3% isoflurane and sensitized with TNBS (Sigma-Aldrich, St Louis, MO, USA) by injecting 100 μl of a 1.0% TNBS solution in PBS subcutaneously one week prior to the administration of the TNBS rectal enema. Seven days post-sensitization, a lubricated 3.5F soft silicon catheter was inserted into the colon to a distance of 3–4 cm to inject a 50 μl enema containing 2.0 mg TNBS in a 50% ethanol/1X PBS solution. Following enema, mice were held inverted for 30 seconds before returning to the cage. Mice were weighed daily, and three days post-enema, mice were euthanized, colons removed, colon length measured, and colonic tissue prepared for histological analysis following fixation in 10% neutral buffered formalin. Hematoxylin/eosin staining was performed on formalin-fixed paraffin-embedded colonic sections and analyzed for inflammation, crypt hyperplasia, ulcerations, bowel wall thickening, and edema blinded for treatment group. Each histological parameter was scored 0 to 1 with a maximum score of 5.

Immunohistochemistry
Slides were placed on the Leica Bond Max IHC stainer. All steps besides dehyration, clearing and cover-slippping are performed on the Bond Max. Slides were deparaffinized. Heat induced antigen retrieval was performed on the Bond Max using their Epitope Retrieval 2 solution for 20 min. The slides were incubated with anti-FOXP3 monoclonal antibody for 1 h at a 1:100 dilution followed by the visualization with Bond Polymer Refine detection system. Finally, slides were dehydrated, cleared and cover-slippped.

Luminex
Simultaneous analysis of multiple cytokines and chemokines were assessed in plasma obtained from humanized mice using Luminex (MilliporeSigma, Burlington, MA, USA).

Statistical Analysis
Statistical analysis was carried out using GraphPad Prism 8 software. P values were calculated using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test and P < 0.05 was considered statistically significant.

RESULTS
Treg Cell Expansion and Protection Against TNBS-Induced Colitis Is Independent of HLA-Restriction
Recent work has demonstrated the efficacy of LD IL-2 as a therapeutic for several immune-mediated diseases (20–23). Previously, we reported that LD IL-2 may be an effective therapy to treat IBD based on our results whereby LD IL-2 induced human T<sub>reg</sub> cell expansion and attenuated experimental colitis in immune-humanized NSG mice expressing human HLA-DQ8 in a setting devoid of murine MHCII (28). To directly investigate the requirement of T<sub>reg</sub> cell HLA restriction for LD IL-2-mediated protection, we employed standard immune-humanized NSG mice purchased from The Jackson Laboratory. All mice were sensitized using a 1% TNBS solution injected subcutaneously. Mice were divided into 3 cohorts with one cohort receiving daily intraperitoneal injections of PBS and another with daily injections of 10,000 IU IL-2. After seven days, mice injected with PBS or LD IL-2 received a rectal challenge of TNBS in a 50% ethanol solution to induce colonic inflammation (Figure 1A). The third cohort that did not receive PBS or LD IL-2 was given an enema containing 50% ethanol as a vehicle control. Mice were weighed daily for three days. While there was no difference in mortality between the LD IL-2 treated or PBS treated groups (Figure 1B), humanized NSG mice treated with LD IL-2 exhibited accelerated recovery from TNBS-induced weight loss compared to mice treated with PBS as a control (P < 0.05) (Figure 1C). Colitis induction in mice often elicits a decrease in colon length that can be used as a prognosticator of inflammation. Therefore, we measured colon length at the experimental endpoint and found that LD IL-2 prevented TNBS-induced colonic shortening compared to PBS (P < 0.05) (Figure 1D). To determine the degree of inflammation microscopically, formalin-fixed paraffin-embedded (FFPE) distal colonic sections were stained with hematoxylin and eosin and scored in a blinded fashion. Although all mice receiving TNBS developed colitis, those receiving LD IL-2 exhibited reduced inflammation and injury compared to PBS (P < 0.001) (Figure 1E) that was similar to what we previously observed when CD4<sup>+</sup> T cells were HLA restricted (28). As expected, NSG mice lacking human immune cells were not protected by LD IL-2 treatment (Supplemental Figure 1). Given the effect of LD IL-2 on T<sub>reg</sub> cell expansion, we performed flow cytometric analysis on leukocytes isolated from the blood and spleen to determine T<sub>reg</sub> cell frequency. LD IL-2 treatment increased T<sub>reg</sub> frequencies in the blood and spleen of humanized mice compared to control and PBS treated mice (P < 0.01; P < 0.001) (Figure 2A). FFPE colon sections stained for FOXP3 showed a significant increase in the number of FOXP3<sup>+</sup> cells in the colon of mice treated with PBS (P < 0.01) (Figure 2B). Interestingly, an increase in colonic FOXP3<sup>+</sup> cells positively correlated with colitis severity (P < 0.001) (Figure 2C).

LD IL-2 Treatment Modifies Select T Cell and NK Cell Subsets
Conventional CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as well as natural killer (NK) cells express the dimeric IL-2R (CD122 and IL-2Rγ) and are competent to bind IL-2, albeit with lower affinity. However, aberrant activation of conventional T or NK cells may elicit unwanted side effects. Therefore, we evaluated CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets for changes in frequency in the blood and spleen of humanized mice treated with TNBS treated with PBS or LD IL-2. We found a slight but statistically significant reduction in naïve CD4<sup>+</sup> T cells (CCR7<sup>+</sup>CD45RO<sup>-</sup>) (P < 0.05) and an increase in effector memory CD4<sup>+</sup> cells (CCR7<sup>-</sup>CD45RO<sup>+</sup>) in the blood with LD IL-2 treatment compared to PBS treated control, and a reduction in naïve CD8<sup>+</sup> T cells following LD IL-2 treatment compared to PBS (P < 0.05) (Figure 3A). In the spleen, we observed a reduction in naïve CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells...
compared to PBS with an increased frequency of effector memory CD4+ similar to that observed in the blood (P < 0.05) (Figure 3B). Human NK cell subsets are often classified into two distinct subsets based on cell surface expression of CD56 and CD16 (35). The CD56^dimCD16^+ population of NK cells participates in the killing of target cells via perforin- and/or granzyme-mediated cytotoxicity (36) and we found a significant increase in this population following LD IL-2 treatment compared to PBS treated (P < 0.05) (Figure 3C). Interestingly, the CD56^brightCD16^dim population of NK cells is described as being immunoregulatory and can proliferate in response to IL-2 (37), yet this population was not altered following LD IL-2 treatment (Figure 3C). Collectively, these data indicate that the majority of CD4^+ and CD8^+ T cell subsets are not significantly
altered by LD IL-2, whereas CD56\textsuperscript{dim}CD16\textsuperscript{bright} NK cells displayed increased frequency following treatment with LD IL-2. We also quantified several human cytokines in distal colon tissue and while LD IL-2 reduced IL-12 production in colon tissue of TNBS-treated mice compared to vehicle treated control (Figure 4), the majority of cytokines evaluated were not significantly altered, suggesting that LD IL-2 may be acting on T\textsubscript{reg} cells in non-colon tissue.

**DISCUSSION**

Here we show that LD IL-2 treatment of immune humanized NSG mice induced human T\textsubscript{reg} cell expansion and was protective against experimental colitis and adds to our previous findings using HLA-restricted humanized mice (28). Interestingly, while the expansion of T\textsubscript{reg} cells in the periphery inversely correlated with disease severity, increased T\textsubscript{reg} cell numbers in the colonic lamina propria positively correlated with disease activity. Given that effector CD4\textsuperscript{+} T cells in humans can upregulate FOXP3 upon activation (38, 39), the true identity of these cells is presently unclear. Importantly, these data show that LD IL-2-induced T\textsubscript{reg} expansion and protection against TNBS-induced colitis was independent of T\textsubscript{reg} cell HLA-restriction. The significance of these findings are that, while some disease models require T cell HLA-restriction in HIS mice (26), evaluation of IBD therapeutics directed at human T\textsubscript{reg} cell expansion and/or function does not require T cell HLA-restriction to attenuate TNBS-induced colitis.

Immunodeficient mice harboring a human immune system are a valuable tractable model for a variety of pre-clinical research applications including leukemia, HIV, and immunoncology. Moreover, the ability to directly assess human therapeutic targets in vivo has the potential to inform novel clinical approaches. In recent years, LD IL-2 has been recognized as an effective therapeutic for treating patients with chronic inflammatory disorders (20–23). Previously we and others showed a requirement for human CD4 T cell selection on human HLA to generate appropriate adaptive immune responses in humanized mice (26, 40). Reasoning that T cell activation is a component of inflammation in the intestine, we initially employed NSG mice expressing human HLA to assess LD IL-2 as a therapeutic to expand human T\textsubscript{reg} cells and suppress experimental colitis (27, 28). While our current study highlights the therapeutic potential for LD IL-2 in treating patients with IBD, one limitation is that colitis induction by TNBS does not require human cells, which is in contrast to what we had previously observed (27). It is likely that the use of ketamine/xylazine, and accompanying extended duration of anesthetization, in the initial study necessitated a lower TNBS
dose that had modest effects on non-reconstituted mice (27). In this study, the rapid recovery from 3% isoflurane as an anesthetic required a higher TNBS dose to induce similar degrees of colonic inflammation that did not require the presence of human cells. Thus, non-humanized NSG mice are also susceptible to TNBS-induced inflammation. Nevertheless, the therapeutic effect of LD IL-2 was dependent on the presence of human immune cells. Whether expanded human T\(_{\text{reg}}\) cells are able to restrict inflammation through direct actions on human immune cells or limit recruitment/activation of murine innate immune cells is not entirely clear. T\(_{\text{reg}}\) cells are known to suppress immune responses through both contact-dependent and -independent mechanisms (41, 42) and murine cells are responsive to many human cytokines.

For patients with IBD, the number of FOXP3\(^+\) cells is often increased within inflammatory lesions (43). Yet, these potent immunoregulatory cells are unable to restore immune homeostasis (44–47). While it is possible that there are still too few regulatory cells to restrain exacerbated inflammatory responses, restricting effector cells in peripheral compartments may play a more significant role in limiting inflammation at least in acute settings. This would be consistent with our data showing peripheral LD IL-2-mediated T\(_{\text{reg}}\) cell expansion was protective. Conversely, increased FOXP3\(^+\) cells in colon tissue correlated with increased disease activity. This suggests that T\(_{\text{reg}}\) cell expansion and suppression of colitis in this model, and perhaps in humans as well, may be more critical in peripheral compartments rather than at the site of inflammation. The increased sensitivity of T\(_{\text{reg}}\) cells for IL-2 compared to conventional T cells is an important property to maintain selectivity. Importantly, we did not observe expansion of conventional CD4\(^+\) or CD8\(^+\) T cells in the blood or spleen with LD IL-2 treatment. We did, however, observe an increase in splenic CD56\(^{\text{dim}}\)CD16\(^+\) NK cells, a population which has been described as being cytolytic, while CD56\(^{\text{bright}}\)CD16\(^+\) NK cells were not altered. This finding was unexpected given that CD56\(^{\text{bright}}\) NK cells are reported to express the high affinity IL-2R and are competent to respond to lower concentrations of IL-2 (48). It has also been reported that NK cells exposed to an immunoregulatory environment can also lower CD56 expression (49), which may be expected in the setting of LD IL-2 given the expansion of regulatory cells and should be considered in future studies and applications.

Collectively, our findings support the therapeutic use of LD IL-2 to expand human T\(_{\text{reg}}\) cells to treat patients with IBD. Based on our model, it appears unlikely that LD IL-2-mediated T\(_{\text{reg}}\) expansion and suppression of immune responses occur in an antigen-specific manner, given that LD IL-2 therapeutic efficacy was observed in humanized NSG mice lacking HLA molecules and therefore limit T\(_{\text{reg}}\) cell TCR-HLA interactions. Thus, immune humanized NSG mice are an ideal system in which to evaluate novel therapeutic strategies targeting human T\(_{\text{reg}}\) cell expansion and/or function in conjunction with experimental colitis as a readout for therapeutic efficacy. Broad commercial availability of immune humanized NSG mice presents a low hurdle that we now demonstrate enables rapid evaluation and innovative approaches aimed at modulating human T\(_{\text{reg}}\) cells for clinical applications.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without reservation.
ETHICS STATEMENT
The animal study was reviewed and approved by The Institutional Animal Care and Use Committee at Vanderbilt University Medical Center.

AUTHOR CONTRIBUTIONS
RT, JJ, JL, ES, KO, and JG conceived and designed the study. RT, JJ, JL, and JG performed humanized mouse experiments. RT, JL, JJ, ES, MA, KW, KO, and JG analyzed and interpreted data. RT, JJ, and JG wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.630204/full#supplementary-material

Supplementary Figure 1 | LD IL-2-mediated protection against TNBS colitis requires human immune cells. Weight loss following TNBS enema in NSG mice devoid of human cells treated with (n = 7) or without (n = 6) LD IL-2.
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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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