This document is provided as a sample research strategy. Some text has been redacted.

Research Strategy

Overview of T Regulatory Adoptive Cell Therapy (ACT) to Treat Autoimmunity

Autoimmunity occurs from a breakdown in immunological tolerance leading to immune attack against self-tissues/organs[1]. $CD4^+Foxp3^+$ T regulatory cells (Treg) are critical for preventing/controlling autoimmune responses[2,3]. Foxp3 is the key transcription factor necessary for the immunosuppressive program in $CD4^+$ T cells and in its absence children quickly succumb to the lethal disease immunodysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX)[4,5]. The occurrence of autoimmune manifestations in IPEX patients demonstrated that Treg are essential to maintain tolerance to self-antigens[5]. Treg utilize a variety of mechanisms to suppress autoimmune T cell responses including disruption of antigen presentation, production of immunosuppressive cytokines, IL-2 consumption and production of immunosuppressive molecules[6,7]. The net result is suppression of TCR signaling pathways that diminish autoreactive T cell activation, thereby preventing/attenuating autoimmunity[6,7]. Treg also suppress NK cells, NKT cells, monocytes, dendritic cells and B cells[6,7]. However, it is not well understood how, when and where Treg suppressive mechanisms are operating in a context-dependent manner in autoimmunity[6,7]. Therapies targeting specific Treg mechanisms would be difficult to administer and would likely have off-target effects. Thus, to harness "natural" immunosuppressive properties of Treg they have been explored as an adoptive cell therapy (ACT).

Conceptually, Treg ACT was proven in multiple mouse models of autoimmunity[8-10]. One of the best studied is the NOD model of type I diabetes where transfer of syngeneic Treg to prediabetic mice conferred protection from diabetes[8]. These early studies prompted investigations into in vitro expansion of Treg for ACT[8]. Success in mice led to clinical trials utilizing autologous in vitro expanded Treg in type I diabetes and graft-versus-host disease (GVHD). Treg ACT was deemed safe and showed some efficacy in GVHD[11,12]. The clinical trials provided proof-of-concept for the enhancement of immune tolerance by increasing baseline Treg numbers. However, utilization of in vitro expanded Treg has a number of drawbacks[13] including:

1) Current Treg isolation techniques lead to varying levels of purity that are well below 100%[3,14].
2) Because Treg numbers in the peripheral blood are low (1-5%) they require in vitro expansion to achieve adequate cell numbers for ACT[3,15].
3) There are no standard methods by to assess the suppressive capacity of in vitro expanded Treg[3].
4) Treg need to be autologous to prevent allorecognition and clearance by the immune system[16,17].
5) Treg need to be autologous to suppress autoimmune responses in an antigen-specific manner[18-21].
6) Treg localization and migration to inflammatory sites is a challenge in the field[14].
7) After transfer a portion of in vitro expanded Treg lose Foxp3 expression and convert to Tconv cells, thereby reducing their potency and generating cells with the potential to drive autoimmunity[14,17].

The above challenges make it clear that new strategies are required to harness the power of Treg. An attractive alternative to in vitro expansion is promoting endogenous Treg proliferation[2,3]. This concept was validated in humans using low dose IL-2 to increase Treg numbers[22,23]. However, NK cell numbers were also increased and depending on the dose so were eosinophils and effector CD4 T cells[22,23]. Thus, major challenges still exist regarding IL-2 dosing to reach and maintain efficacy without unwanted adverse effects[22,23]. An additional drawback to IL-2 therapy is its short plasma half-life, which requires administration every 1-3 days via subcutaneous injection[22-26].

Thus, based on review of rigor in the prior research in regards to Treg ACT, it is clear that new approaches need to be developed to counter its many challenges. **Here, we outline a novel strategy to develop a first-of-its-kind B cell-based universal "off-the-shelf" ACT that will overcome current weaknesses and challenges associated with Treg ACT.** Our strategy is based on our recent identification of a new B cell subset (**B cell IgD low (BD_L)**) that promotes immune tolerance by inducing Treg proliferation thereby sustaining their cell numbers[27]. Its design will enhance BD_L mechanistic properties to increase baseline Treg numbers to boost immunological tolerance. By expanding endogenous Treg, we will overcome issues associated with purity, activity and stability encountered in Treg ACT. Because BD_L mechanisms do not require antigen presentation there is no need for an autologous product. This finding allowed the development of a strategy to develop an off-the-shelf BD_L product by eliminating the expression of MHC class I and II molecules to prevent allorecognition and subsequent deletion. We will also introduce an NK cell inhibition mechanism to prevent killing of MHC class I negative BD_L. All current T cell-based ACT require in vitro expansion and/or genetic manipulation of autologous T cells to generate a limited dosage product that is very expensive ($0.5-1.5 million/treatment) with little control over its efficacy. Our BD_L ACT is designed such that one well-defined

product could be administered multiple times to any patient deemed to benefit from increased Treg immunological tolerance. A BD_L ACT could be used as a stand-alone or a combination therapy. In addition, since it will utilize "natural" immune mechanisms to increase and sustain endogenous Treg, side-effects should be minimal. **Our success has the potential to transform how autoimmunity and other chronic inflammatory diseases are treated with the added benefit of greatly reducing the cost of treatment.**

Discovery of a Novel B Cell Subset that Maintains Immune Tolerance Through Interaction with Treg. Our concept for development of a BD_L-based ACT is based on our discovery that B cells have the capacity to negatively regulate the severity of autoimmunity[28]. Specifically, using the animal model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), we discovered that mice deficient in B cells did not recover from the signs of EAE[28]. This seminal finding sparked the search for regulatory B cell phenotypes. Although IL-10 production by B cells has emerged as a powerful anti-inflammatory mechanism, there is no definitive B cell subset/phenotype that can be used to identify and purify them making them difficult therapeutic targets[29]. Thus, we concentrated our research efforts on discovering IL-10-independent regulatory B cell mechanisms[30]. Utilizing the EAE model, we discovered that the small population of IgM^+ B cells with low/neg IgD expression (**B cell IgD low (BD_L)**) (**Fig. 1**) play a prominent role in Treg homeostasis[27]. We found that mice deficient in BD_L due to genetic ablation (μMT) (**Fig. 2A**) or antibody depletion with anti-CD20 had a significant reduction in Treg numbers (**Fig. 2B**) and did not recover from EAE (**Fig. 2C**)[27,28,31]. Restoration of BD_L in μMT mice restored Treg numbers (**Fig. 2D**) and the mice recovered from EAE (**Fig. 2E**)[27,31]. We found that the increase in Treg was due to increased proliferation as determined by BrdU and Ki-67 staining as well as cell proliferation dye dilution (data not shown)[27,31]. We then asked whether the mature splenic B cell subsets follicular (FO) and marginal zone (MZ) (**Fig. 1**) also exhibited regulatory activity. We found that neither FO (**Fig. 2D**) nor MZ (data not shown) B cells promoted Treg expansion, consistent with their inability to drive recovery from EAE (**Fig. 2E**)[31]. These data indicate that only BD_L exhibit the unique effector function of driving Treg proliferation. Although certain B cell subsets have the capacity to generate iTreg[30], we want to emphasize that BD_L do not utilize that mechanism. **BD_L drive Treg proliferation not Foxp3 induction[27,31].** Our results are consistent with studies whereby Treg ACT attenuated EAE severity[32,33]. In addition, these cumulative results support the concept that there is a threshold level of Treg necessary to control autoimmune responses and that increasing Treg numbers with a BD_L-based ACT is a viable strategy to attenuate the severity of autoimmune disease[34]. **Bolstering this concept is our finding that BD_L do not require MHC class II expression to induce Treg proliferation (data not shown)[31]. Eliminating antigen presentation paves the way for the development of a "universal" BD_L-based therapeutic that could be administered to any patient regardless of MHC haplotype.**

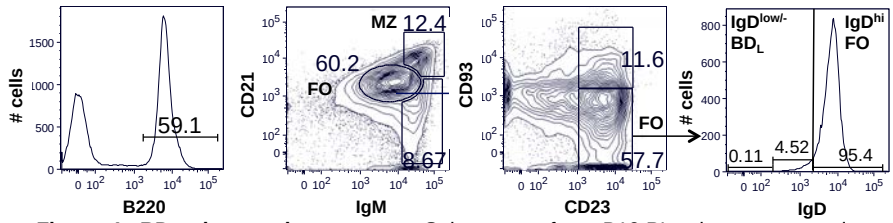


Figure 1. BD_L phenotyping strategy. Splenocytes from B10.PL mice were gated on $B220^+$ followed by gating for follicular (FO) (IgM^+CD21^{int}) and marginal zone (MZ) ($IgM^{hi}CD21^{hi}$) B cells. Mature $CD23^+CD93^-$ FO B cells were characterized for IgD expression and subdivided into $IgD^{low/-}$ BD_L and IgD^{hi} FO B cells.

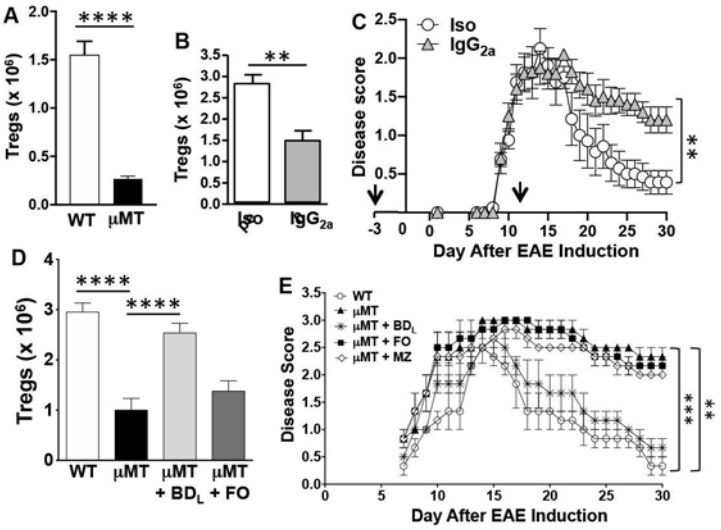


Figure 2. B cell deficiency results in loss of Treg and chronic EAE, both of which are corrected with B cell transfer. B10.PL mice deficient in B cells by genetic disruption (μMT) (A,D,E) or following administration of anti-CD20 IgG_{2a} or its isotype control (250 mg) (B,C) on days -3 and 11 were utilized. $CD4^+Foxp3^+$ Treg were quantitated in the spleen in unmanipulated mice (A) or on day 14 (B). EAE was induced by the adoptive transfer (i.v.) of MBP-specific encephalitogenic T cells (1×10^6) into sublethally irradiated mice (C,E). Clinical signs of EAE were assessed daily using a numerical scale from 0-5 as follows: 0, no disease; 1, tail weakness and/or wobbly walk; 2, hind limb paresis; 3, hind limb paralysis; 4, hind and forelimb paralysis; and 5, death or euthanasia. BD_L, FO and MZ B cells purified from naïve mice and 5×10^6 cells were co-transferred into μMT mice and Treg were quantitated in the spleen 10 days later (D) or EAE was induced two days later (E). WT and μMT control mice received PBS. N = 6-7 (A), 3-4 (B), 6-10 (C), 6 (D) and 3 (E). **p<0.01; ***p<0.001; ****p<0.0001.

BD_L Activity in Humans. A challenge in biomedical research is translating findings from animal models to human disease. With that as our goal, we conducted experiments to identify human BD_L. We found that human B cells with the same $IgM^+IgD^{low/-}$ phenotype (**Fig. 3A**), as that described in mice (**Fig. 1A**), also promoted Treg expansion (**Fig. 3B**)[27]. BD_L from both human spleen (data not shown) and peripheral blood (**Fig. 3B**) exhibited BD_L activity[27]. **The discovery that BD_L exist in humans provided the requisite rationale for their development as an ACT.**

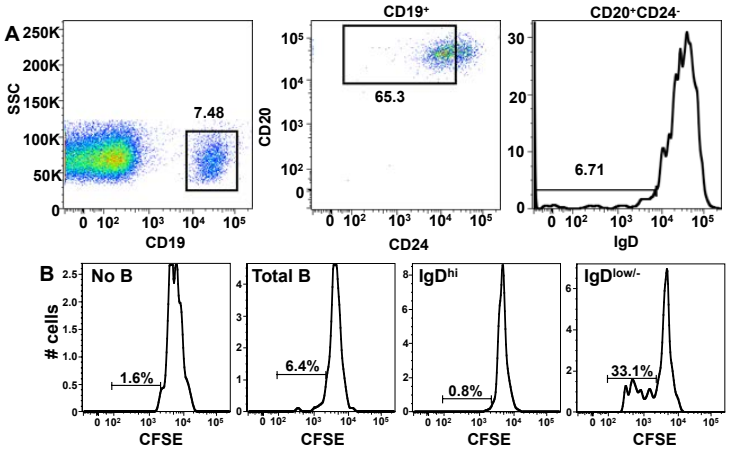


Figure 3. Human $IgD^{low/-}$ B cells induce the proliferation of human Treg in vitro. Human peripheral blood total B cells ($CD19^+$), IgD^{hi} ($CD19^+CD20^+CD24^-IgD^{hi}$) B cells and IgD^{low} ($CD19^+CD20^+CD24^-IgD^{low/-}$) B cells were FACS purified (A). Treg ($CD4^+CD25^{hi}$) were FACS purified from the same donor, CFSE labeled and 0.5 x 10^5 cells were cultured alone or with 1 x 10^5 total, IgD^{hi} or $IgD^{low/-}$ B cells in the presence of plate-bound anti-CD3 (10 mg/ml) and anti-CD28 (10 mg/ml). After culture, the cells were stained with CD4 and the percentage of proliferating Treg cells was determined by flow cytometry (B). Data shown are one representative experiment of two.

Enhancement of BD_L Mechanisms. When we investigated potential mechanisms whereby BD_L drive Treg proliferation, we focused on glucocorticoid-induced tumor necrosis factor receptor ligand (GITRL) because engagement with its receptor GITR was shown to induce Treg proliferation in the absence of cognate interactions[35]. Antibody blocking of GITRL on BD_L abrogated their ability to induce Treg proliferation (**Fig. 4A**) and drove recovery from EAE (**Fig. 4B**). One goal of Treg therapy is to increase their numbers above the steady state to enhance their suppressive activity. Thus, we determined whether GITRL overexpression in BD_L could be leveraged to enhance their regulatory activity. This was accomplished by utilizing GITRL transgenic (GITRLtg) mice with expression restricted to B cells, which increased its expression by ~4-fold (**Fig. 4C**)[36]. We confirmed that GITRLtg mice have increased numbers of Treg (**Fig. 4D**) and attenuated EAE disease severity (**Fig. 4E**)[36]. The increase in Treg in GITRLtg mice was sustained throughout the EAE disease course with GITRLtg mice having ~19-fold more splenic Treg as compared to WT on day 30 (**Fig. 4F**). We then determined that adoptive transfer of GITRLtg BD_L significantly enhanced Treg expansion by ~2-fold as compared to WT BD_L following transfer into μMT mice (**Fig. 4G**). Importantly, GITRL overexpression did not endow FO or MZ B cells with the capacity to promote Treg expansion (**Fig. 4G**). Since GITRL has also been reported to promote the proliferation of T conventional (Tconv) cells[37], we quantitated their cell numbers after BD_L transfer. Interestingly, while Tconv numbers were increased in the GITRLtg mouse as compared to WT (data not shown), transfer of GITRLtg BD_L did not increase their numbers as compared to WT BD_L (**Fig. 4H**). This is consistent with Tconv cells having lower GITR expression as compared to Treg, which we confirmed (data not shown)[38].

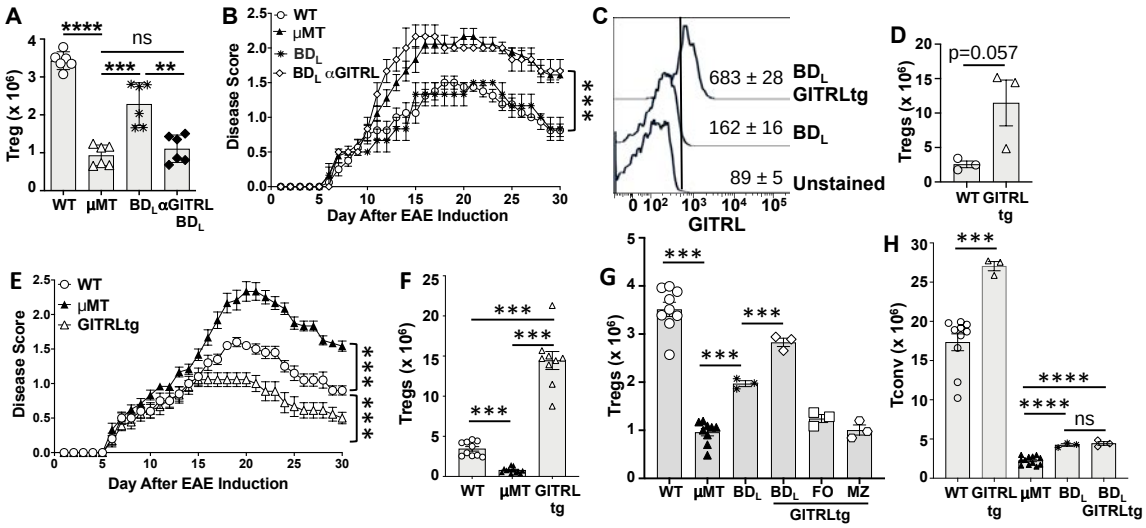


Figure 4. BD_L induce Treg expansion in a GITRL-dependent manner. μMT mice were reconstituted with WT or anti-GITRL blocked BD_L (5 x 10^6) and the absolute number of Treg was determined in the spleen 10 day later (A) or EAE was induced as for Fig. 2A (B). GITRL expression by BD_L (C) and splenic Treg numbers (D) were quantitated in WT and GITRLtg mice by flow cytometry. EAE was induced in WT, μMT and GITRLtg mice as for Fig. 2A (E) and the absolute number of splenic Treg was quantitated on day 30 (F). G,H) BD_L from WT; or BD_L, FO or MZ B cells from GITRLtg mice (5 x 10^6) were adoptively transferred into μMT mice and the absolute number of splenic Treg (G) and Tconv cells was determined 10 days later. A, D, and F-H each data point represents one mouse. N=3-9 (B), 4 (C), and 9-12 (E). **p<0.01, ***p<0.001, ****p<0.001. ns= not significant.

For BD_L to be an effective ACT, their numbers would need to be sustained during autoimmune attacks. We examined this question by adoptively transferring BD_L into ∝MT mice and then tracking them during EAE. As shown in **Fig. 5A**, we could detect BD_L ($IgD^{low/-}$) at onset (d7), peak (d18) and after recovery (d30), indicating that they are sustained during autoimmunity[27]. The sustained presence of BD_L was correlated to a significant increase in Treg in ∝MT mice that received BD_L on day 30 of EAE (**Fig. 5B**). **Collectively, our preliminary studies provide a proof-of-concept that BD_L genetically engineered to overexpress GITRL could be used to enhance their regulatory activity. In addition, our data indicate that a sustainable BD_L ACT could be generated to specifically target Treg without increasing Tconv cell numbers.** Although GITR is considered a therapeutic target for Treg expansion, to date, its potential has not been harnessed[2,38].

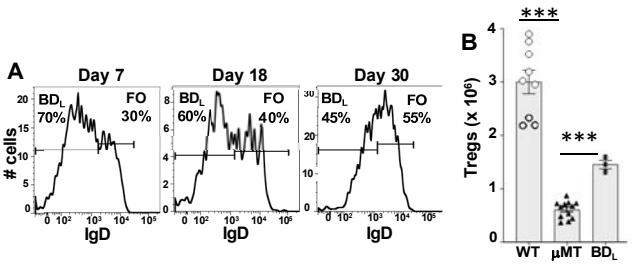


Figure 5. BD_L are a stable phenotype after transfer in EAE. A,B) BD_L adoptive transfer and EAE was performed as for Fig. 2. A) In µMT mice that received BD^L (5 x 10^6) on days 7, 18 and 30 of EAE, $B220^+$ lymphocytes were gated for IgM^+CD23^+ and the percent BD_L ($IgD^{low/-}$) and FO (IgD^{hi}) B cells was determined by flow cytometry. 1 experiment of 2 is shown. B) On day 30 of EAE Treg absolute cell numbers were quantitated in the spleen of WT, µMT and µMT mice + BD_L mice by flow cytometry. Each data point represents one mouse. ***p<0.001.

BD_L are a Unique B Cell Subset. Each mature B cell subset has a unique effector function. FO B cells are the primary contributors of isotype-class switched antibodies[39] and MZ B cells provide front-line defense against blood-borne pathogens[40]. Although only BD_L can induce Treg proliferation, function alone is not sufficient to conclude they are a unique B cell subset. Immature B cells exist the bone marrow (BM) and enter the spleen and differentiate through the transitional (T) 1 then T2 stages (**Fig. 6A**). At the T2 stage B cells make a fate decision leading to differentiation into mature MZ B cells or mature FO B cells (**Fig. 6A**). To determine where BD_L fit into B cell development, we followed their emergence in the spleen following BM transplantation (T). T2 B cells emerged on day 15 and peaked on day 17 (**Fig. 6B**). The finding that FO B cells peaked on day 21 confirmed that they develop from the T2 stage (**Fig. 6B**)[41]. BD_L exhibited a similar developmental kinetics as FO B cells, indicating BM derivation and development from the T2 stage (**Fig. 6B**)[27]. These results were confirmed using B cell reconstitution following sublethal irradiation and T2 adoptive transfer studies (data not shown)[27]. To determine whether BD_L are a unique B cell subset, we performed RNAseq analysis comparing the transcriptomes of BD_L, FO and MZ B cells. Using principal component (PC) analysis plotted in three-dimensions (3D), as expected, FO and MZ B cells clustered differentially in all three dimensions (**Fig. 6C**)[27]. A similar result was obtained when BD_L and MZ were compared (**Fig. 6C**)[27]. When we compared BD_L to FO B cells they clustered similarly in PC1 and PC2, but clustered independently in PC3 (**Fig. 6C**)[27]. These data indicate that BD_L are a new B cell subset independent from FO and MZ B cells. **Since BD_L are a novel B cell subset their unique tolerogenic activity can be harnessed without compromising effector functions of the other mature B cell subsets.**

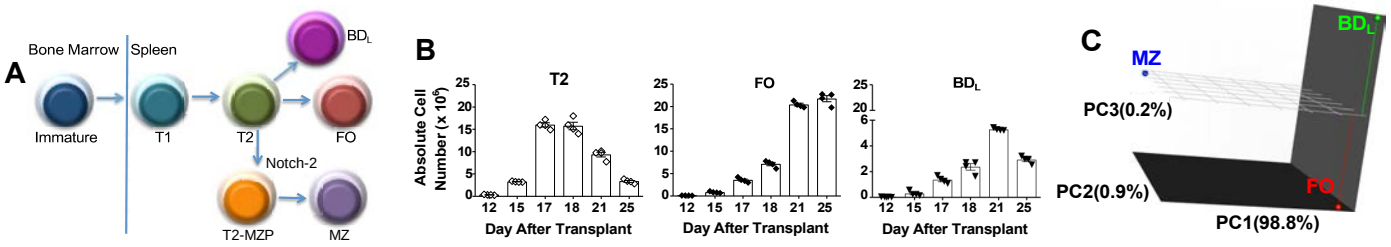


Figure 6. BD_L are a distinct B cell subset emerging from the T2 stage. A) A diagram showing B cell development in the spleen indicating that BD_L emerge from the T2 stage similar to FO and MZ B cells is shown. B) Mice were lethally irradiated (950 rad) and transplanted with syngeneic BM and the emergence of T2 ($B220^+IgM^{hi}CD21^{low/-}CD23^+CD93^+$), FO ($B220^+IgM^+CD21^{int}CD23^+CD93^-IgD^{hi}$) and BD_L ($B220^+IgM^+CD21^{int}CD23^+CD93^-IgD^{low/-}$) was tracked in the spleen on the indicated days by flow cytometry and the absolute number of cells was calculated. Each symbol represents one mouse. C) PC analysis was performed on the log2-transformed FPKM, with the % variance encompassed within each PC shown. The distribution of BD_L (green), FO (red) and MZ (blue) within the 3D plot are shown. N = 3.

Chemokine Receptor Driven Localization in the Spleen is Required for BD_L Regulatory Activity. While GTRL is essential for BD_L regulatory activity, a mechanism must exist whereby BD_L preferentially localize to areas in the spleen enriched in Treg. In that regard, lymphocyte migration in the spleen is tightly controlled by a combination of chemokine receptor expression levels and responsiveness to ligands. CCR7 drives migration into the T cell zone in response to CCL21[42,43] and CXCR5 directs migration into the B cell follicle in response to CXCL13[44]. FO B cells express higher levels of CXCR5 (**Fig. 7A**) than CCR7 (**Fig. 7B**). This expression pattern

leads to higher migration to CXCL13 (**Fig. 7C**) as compared to CCL21 (**Fig. 7D**). The imbalanced chemokine response towards CXCL13 drives FO B cells into the B cell follicle[44]. BD_L express CXCR5 (**Fig. 7A**) and migrate to CCXL13 (**Fig. 7C**) similar to FO B cells. However, as a population, BD_L skew towards higher expression of CCR7 (**Fig. 7B**), which is consistent with increased migration to CCL21 (**Fig. 7C**), as compared to FO B cells. We next tested the requirement for BD_L CXCR5 and CCR7 expression for their ability to expand Treg in vivo. Neither CXCR5 (**Fig. 7E**) nor CCR7 (**Fig. 7F**) were absolutely required for BD_L to induce Treg expansion in vivo, as compared to μMT mice. However, the absence of either receptor significantly reduced the number of recovered Treg (**Fig. 7E,F**). **These data demonstrate that tuning of chemokine receptor responsiveness and subsequent BD_L localization can enhance BD_L activity.**

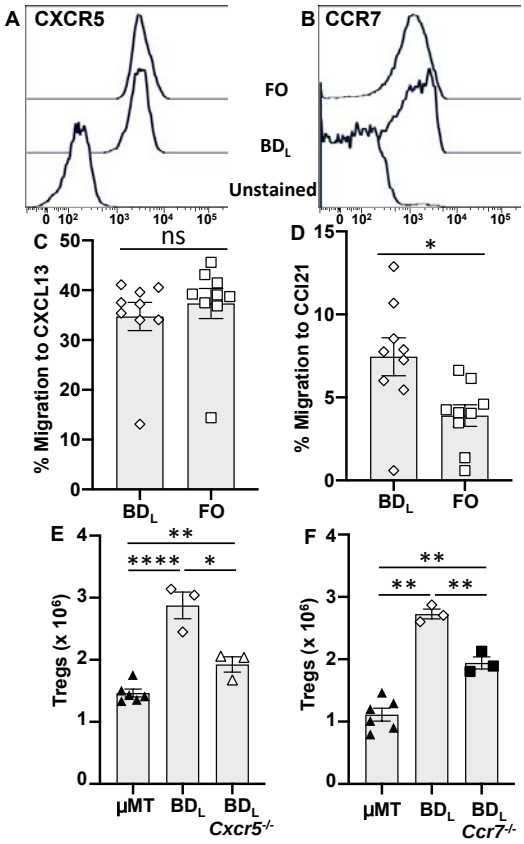


Figure 7. BD_L exhibit a distinct chemokine receptor expression and migration pattern from FO B cells and require CXCR5 and CCR7 expression for regulatory activity. A,B) BD_L and FO B cells were phenotyped for expression of CXCR5 (A) and CCR7 (B) by flow cytometry. C,D) Splenic BD_L and FO B cellswere placed in the upper chamber of a 5 mm Transwell and allowed to migrate toward medium plus CXCL13 (C) or CCL21 (D) at the indicated concentrations for 3 h. Migrated cells were collected from the lower chamber and the percent migrated cells was determined by flow cytometry. E,F) WT (E.F), *Cxcr5*$^{-/-}$ (E) or *Ccr7*$^{-/-}$ (F) BD_L (5 x 10^6) were adoptively transferred into μMT mice and the absolute number of Treg was determined 10 days later. N= 5 (A,B), 9 (C,D) and 3-6 (E,F). *p<0.05, **p<0.01; ****p<0.0001. ns = not significant.

Because BD_L have increased responsiveness to the T cell zone chemokine CCL21, we hypothesized that they would localize to the T:B border. This concept is supported in T follicular helper cell positioning by balanced expression of CXCR5 and CCR7[45,46]. To test our hypothesis, we utilized a four-color immunofluorescence strategy staining for CD4 and Foxp3 to identify Treg, IgM for total B cells and IgD for FO B cells. BD_L were identified by expression of IgM, but not IgD. In the top panel of **Fig. 8** the arrows point to B cells (IgM^+, yellow) that are in contact with Treg ($CD4^+$ (blue) $Foxp3^+$ (green) at the border of the T cell zone and B cell follicle. In the bottom panel of **Fig. 8**, B cells interacting with Treg are IgD, as indicated by absence of red fluorescence. **These cumulative results indicate that altering chemokine receptor levels on BD_L to optimize their localization to the T:B border could enhance their interactions with Treg.**

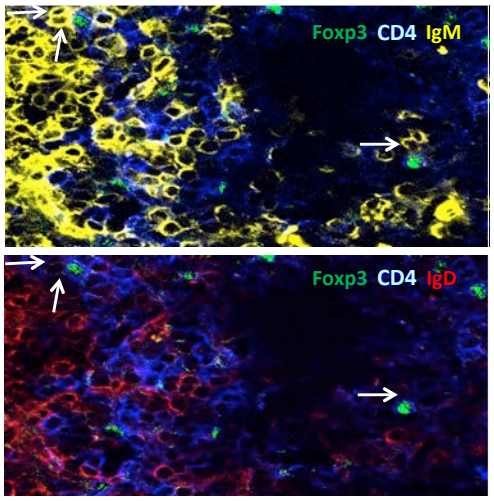


Figure 8. BD_L co-localize with Treg at the border of the T and B cell zones in the spleen. Splenic frozen sections from C57BL/6 mice were stained with the antibodies Foxp3 AF4888 (green), CD4 BV421 (blue), IgM AF647 (yellow) and IgD AF594 (red) and imaged for 4-color immunofluorescence. The top and bottom panels are the same image with both showing Foxp3 and CD4 staining and the top panel IgM and the bottom panel IgD. The arrows indicate BD_L:Treg interactions. The image is 60x.

Our goal of developing a BD_L cellular therapeutic that can increase baseline Treg numbers long-term is based on our novel discoveries, which provided the insight that B cells play an important role in immunological tolerance by maintaining Treg homeostasis. This information will be leveraged to generate a BD_L-based ACT that will be genetically engineered so that it can be universally administered to any patient. This process will utilize state-of-the-art techniques/strategies in gene editing (CRISPR/Cas9) and ACT. Current ACT are generated for a single individual making them difficult and expensive to generate and limits the number of treatments possible. Our strategy would eliminate all of the drawbacks of Treg ACT because it would target endogenous Treg allowing them to function naturally. By accomplishing our goal, we would revolutionize Treg-based ACT because every candidate patient could be treated with the same off-the-shelf product. A "universal" product would dramatically reduce the cost of ACT purposed to boost Treg numbers. While in principal a universal BD_L-based ACT could be used to treat a large number of autoimmune or chronic inflammatory

diseases, in practice that may not be the case. In the Approach, we will discuss those autoimmune diseases that are most likely to benefit from BD_L ACT.

Approach

Our plan to generate a BD_L ACT will be accomplished in a two-pronged approach. In Objective 1, we will build and test a mouse BD_L (mBD_L) prototype. Simultaneously, in Objective 2, we will begin generating a human BD_L (hBD_L) ACT incorporating knowledge gained from the mouse prototype into its design. **In doing so, per FOA instructions, a detailed experimental plan and substantial preliminary data are not being provided. Instead, we will provide the underlying logic/rationale for why we are pursuing this project in the manner designed.** Statistical support for all studies will be provided by [redacted]).

Objective 1. Generation of a mBD_L prototype optimized to induce Treg proliferation and rendered invisible to the immune system

Step-by-step list of procedures to generate a mBD_L prototype

1) Select a parent B cell with self-renewing capacity
2) Screen for mBD_L activity in vitro
3) Screen for mBD_L activity in vivo
4) Express or overexpress proteins required for mBD_L activity
5) Gene editing to alter expression of proteins required for mBD_L activity
6) Gene editing to knockout MHC class I and II expression
7) Strategy to inhibit NK cell lysis
8) Test mBD_L functionality in a therapeutic model of EAE

Step 1. Select a parent B cell with self-renewing capacity. B cells undergo minimal homeostatic expansion once they mature and their proliferation cannot be sustained after activation. Thus, a naïve BD_L cannot be used as the parent cell. In the course of selecting a parent cell, we found that B cell chronic lymphocytic leukemia (CLL) share characteristics with BD_L[47]. Like BD_L, CLL are typically IgM^+ and IgD^+ or IgD^-[47-49]. Several studies have reported high levels of GITRL expression by CLL consistent with increased numbers of Treg[50-54]. Thus, we searched the literature and identified and acquired three mouse CLL lines of interest - EMC2, EMC4 and EMC6[55]. EMC2 and EMC4 are from *IgH.TEμ* mice on a mixed C57BL/6 (B6) x 129 background and EMC6 is from *Aicd*$^{-/-}$ B6 x 129 mice[55]. Of the three lines, only EMC4 is $IgM^+IgD^{low/-}$, which we confirmed by flow cytometry (**Fig. 9**). EMC4 also expresses GITRL, CXCR5 and CCR7 (**Fig. 9**). Since EMC4 exhibits all of the required BD_L cell surface characteristics ($IgM^+IgD^{low/-}GITRL^{low}CCR7^+CXCR5^+$) it was chosen as the parent cell for the mBD_L prototype.

If EMC4 is not found to be a suitable parent cell, we will then investigate whether one of the other two cell lines would be appropriate. If none of the CLL lines is found to be appropriate, we will search for additional mouse transformed B cell lines to test. For simplicity, hereafter the parent mBD_L prototype will be referred to as EMC4.

Step 2. Screen for mBD_L activity in vitro. The primary characteristic required for the prototype BD_L is the ability to induce Treg proliferation via GITRL. This will be tested using a novel in vitro assay developed by us to test human BD_L activity (**Fig. 3**)[27]. Since both C57BL/6 and 129 mice are H-2^b, we can utilize B6 mice for these studies without concern of TCR allogenic recognition of EMC4. For the in vitro assay, FACS purified Treg from Foxp3^{EGFP} reporter mice will be labelled with a cell proliferation dye and co-cultured with EMC4 and after seven days Treg proliferation will be determined by flow cytometry, as we described[27,31]. Ki-67^{RFP} reporter mice will be utilized as an alternative to the cell proliferation dye because it is only expressed during the cell cycle[56]. $EGFP^+RFP^-$ Treg (nonproliferating) will be utilized for the assay and proliferation will determined by the percent RFP^+EGFP^+ Treg. We are currently generating Foxp3^{EGFP} x Ki-67^{RFP} mice and have validated the RFP reporter. Since EMC4 is transformed its proliferation will be attenuated by irradiation or mitomycin-c, so that it doesn't overtake the culture. The requirement for GITRL to induce Treg proliferation will

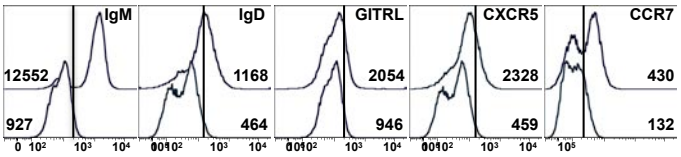


Figure 9. EMC4 express BDL markers. The mouse CLL cell line EMC4 was phenotyped for expression of IgM (A), IgD (B), GITRL (C), CXCR5 (D) and CCR7 (E) by flow cytometry (top histogram). Back ground staining is shown in the bottom histogram. The mean fluorescence intensity is provided.

be determined by GITRL antibody blocking (**Fig. 4A**). If Treg proliferation is observed, we will move to step 3 (BD_L activity in vivo). If Treg proliferation is not observed, we will move to step 4 (manipulate proteins required for BD_L activity).

Step 3. Screen for mBD_L activity in vivo. Once we have confirmed that EMC4 induces Treg proliferation in a GITRL-dependent manner, we will test its BD_L activity in vivo. EMC4 will be sublethally irradiated and adoptively transferred into B6μMT mice and Treg and Tconv numbers will be quantitated 10 days later**.** Proliferation will be assessed by Ki-67 expression[31]. We are currently generating Ki-67^{RFP}μMT mice to be used as recipients to monitor increased Treg proliferation using the Ki-67 reporter as an alternative approach. Treg proliferation will be confirmed using a second in vivo assay in which EMC4 will be co-transferred with FACS purified $Foxp3^{EGFP}$ Treg that are proliferation dye labelled or from Ki-67^{RFP} into μMT mice, and seven days later Treg proliferation will be measured by flow cytometry[27,31]. Because irradiation will upregulate danger signals that could lead to EMC4 clearance an alternative strategy will be mitomycin-c treatment.

Interestingly, CCR7 is expressed as a bimodal distribution by both mouse BD_L (**Fig. 7B**) and EMC4 (**Fig. 9**). Since CCR7 expression is required for migration to the T cell zone and full BD_L activity, we will FACS purify $CCR7^+$ and $CCR7^-$ EMC4 and culture them to determine whether the expression profile of CCR7 is stable post-sort. If it is, we will test the ability of EMC4 $CCR7^+$ and $CCR7^-$ lines to induce Treg proliferation in vivo. If the CCR7 phenotype is not stable, we will then single cell clone $CCR7^+$ and $CCR7^-$ EMC4 and repeat the in vivo Treg proliferation assay. If Treg proliferation is observed, we predict it will be in the $CCR7^+$ population and we will move to step 6 (KO MHC genes). If proliferation is not observed, we will move to step 4 (manipulate proteins required for BD_L activity).

Step 4. Express or overexpress proteins required for mBD_L activity.
GITRL. Although EMC4 expresses GITRL at a low level (**Fig. 9**), its expression may not be high enough to induce Treg proliferation. To overcome this, we will transduce EMC4 with lentiviral vectors containing GITRL driven by a strong ubiquitous promoter. EMC4 with increased GITRL expression will be FACS purified and single cell cloned. Individual clones will be tested for BD_L activity in vivo. We will also generate EMC4 lines with varying GITRL expression levels, to determine the expression level that provides maximal Treg proliferation/expansion. The clone driving maximal Treg proliferation will be moved forward in the pipeline.

Chemokine receptors. For step 1, we characterized EMC4 CXCR5 and CCR7 expression levels. Here, we will determine EMC4 migration levels to CXCL13 and CCL21, as for **Fig. 7C and D**, respectively. We will test EMC4 clones expressing various level of CCR7 generated in Step 3. If migration to CXCL13 and/or CCL21 are low, we will then increase their expression genetically as for GITRL. We will repeat the migration assay and assess BD_L-induced Treg proliferation in vivo. We will tweak CXCR5 and CCR7 expression levels using strong to weak promoters in the lentiviral vectors to achieve maximal Treg proliferation. As an alternative, cell signaling assays to measure the strength of chemokine receptor signaling to their ligands could be performed. Localization studies will also be conducted to determine where the genetically modified mBD_L localize in the spleen (**see Fig. 8**). If mBD_L localize to the T:B border and induce Treg proliferation, we will move to step 6 (KO MHC genes). If not, we will move to step 5 (proteins required for BD_L activity).

Step 5: Gene editing to alter expression of proteins required for mBD_L activity. In step 4, we may not achieve the balanced CXCR5:CCR7 signaling required for optimal in vivo migration of EMC4 using a transgenic approach. If that occurs, we will implement the alternative strategy of CRIPR/Cas9 gene editing to knockout CXCR5 and/or CCR7 and then reintroduce them using lentiviral vectors containing promotors that range from weak - strong to vary their expression level. EMC4 clones generated will be evaluated by flow cytometry and cell migration assays to determine chemokine expression levels that drive optimal chemokine responsiveness, respectively. The EMC4 clones that provide results most similar to WT BD_L will be tested in the in vivo proliferation assay. CRISPR/Cas9 gene editing will be conducted in collaboration with [redacted] [57,58]. Lentiviral vector design and generation will be conducted in collaboration with [redacted].

At the end of step 5, the goal is to have in hand a prototype mBD_L that induces Treg proliferation in vivo. While

all of the strategies outlined above are feasible, there is risk that we will not be successful. This would likely be due to an additional factor(s) required for mBD_L activity. Using single cell RNAseq comparing BD_L to FO B cells, we have identified candidate cell surface proteins that we are currently investigating. These include CD24 and CD83. CD24 can function as an adhesion molecule facilitating cell aggregation through homophilic interactions and is important for T cell homeostasis[59,60,61]. Thus CD24 homophilic interactions between BD_L and Treg could be a second signal in addition to GITR to induce Treg proliferation. CD83 expression has been shown to promote the longevity of lymphocytes following adoptive transfer, suggesting that increasing its expression on hBD_L could enhance their cell survival[62].

Step 6: Gene editing to knockout expression of MHC class I and class II molecules. To make a universal off-the-shelf ACT it must be compatible with all MHC haplotypes to prevent allorecognition and removal by the immune system. One strategy is to make the mBD_L invisible to the immune system by knocking out expression of all MHC class I and II molecules. All classical and non-classical (including CD1) MHC class I proteins require beta 2 microglobulin (B2m) for expression on the cell surface[63]. Thus to knockout expression of MHC class I molecules, CRISPR/Cas9 will be used to target *B2m*. Transcription of MHC class II proteins requires the class II, major histocompatibility complex, transactivator (Ciita)[64], which will be targeted with CRISPR/Cas9[65]. Loss of protein expression will be confirmed by flow cytometry and Western blotting. The Dittel laboratory is currently learning the CRISPR/Cas9 technology and has designed and is testing guide RNAs for mouse *B2m* in collaboration with [redacted].

We have found that BD_L do not require cognate interactions mediated by MHC class II to induce Treg proliferation, thus deletion of all MHC genes will not alter its effector function[31]. Deletion of all MHC genes will also be advantageous because that will circumvent the BD_L ACT from presenting antigen that could led to its targeting by the immune system or its playing a role in adaptive immune responses.

Step 7: Strategy to inhibit NK cell lysis. One consequence of deleting all MHC class I molecules is that they will be recognized by NK cells and lysed[66]. However, HLA-E and HLA-G inhibit NK killing in humans[67,68]. Their mouse homologs are Qa1 and Qa-2, respectively[69,70]. To prevent allorejection, EMC4 cells will be engineered to express Qa1 and/or Qa2. The Qa1/Qa2 gene will be tethered to B2m to facilitate their expression on the cell surface. This strategy has been used successfully for HLA-G[71]. NK lysis will be monitored using in vitro killing assays[72]. In vivo clearance of the mBD_L prototype will be assessed in all H2 haplotypes. If NK killing is prevented, Treg proliferation will be assessed in vivo.

Step 8: Test BD_L functionality in a therapeutic model of EAE. Once we have a mBD_L prototype in hand it will be utilized in animal models to determine whether it is effective at attenuating disease severity. First, we will utilize EAE and will monitor disease scores and quantitate Treg numbers and their proliferation level ($Ki\text{-}67^+$) throughout the disease course, as for **Fig. 4**. We will also track mBD_L cell numbers. The mBD_L will be irradiated or mitomycin-c treated. The results in EAE will be confirmed in contact hypersensitivity (CHS), since we have shown that BD_L also reduces its severity in μMT mice[27]. If the prototype mBD_L induces Treg proliferation, we predict that disease severity will be attenuated in both EAE and CHS. We will test mBD_L activity in both male and female mice to confirm rejection will not occur in a sex-dependent manner. If rejection due to sex is indicated, we will determine the sex of the EMC4 clone, which was not disclosed in the original article, and determine if rejection is due to female recognition of male antigens, such as the H-Y antigen[73].

The mBD_L prototype that induces the highest level of Treg proliferation and/or attenuates disease severity the greatest will be tested in an animal model that mimics how it would be utilized in humans. We will deplete endogenous B cells prior to adoptive transfer of mBD_L. This strategy is based on rituximab, an anti-CD20 therapy for non-Hodgkin's lymphoma, which also leads to depletion of endogenous B cells[74]. It was subsequently found that B cell depletion with rituximab in autoimmune diseases including rheumatoid arthritis, pemphigus vulgaris, myasthenia gravis, multiple sclerosis and others showed efficacy in reducing disease severity and progression[75-78]. **Depletion of B cells with anti-CD20 depleting therapy would have a dual benefit of lymphodepleting pathogenic B cells while providing space for the BD_L ACT to occupy.** For this strategy to work in humans the hBD_L would need to lack CD20 expression (see Objective 2). For our mouse strategy, we obtained human CD20tg mice in which B cell depletion will be achieved using anti-human CD20[79]. In the laboratory, we currently have three anti-human CD20 antibodies, one of which is rituximab. Using our extensive experience in anti-CD20 B cell depletion in mice, we have developed a strategy in which human CD20tg mice will be administered anti-human CD20 and 14 days later mBD_L will be adoptively

transferred. We will experimentally determine the half-life and localization of mBD_L. We will also monitor the return of endogenous B cells and quantitate Treg numbers. Once we have established a model whereby mBD_L are engrafted long-term and/or Treg numbers are boosted long-term, we will induce EAE and monitor for attenuated disease severity. We will also test mBD_L activity in CHS and dextran sulfate sodium-induced colitis mouse models, which are established in the Dittel lab[27,80]. Finally, we will test mBD_L in animal models of disease not on the B6 background including graft-versus-host disease (GVHD) and type I diabetes (NOD) by recruiting collaborators from MCW[81,82]. The results from these studies will provide important information as to which human diseases are most likely to benefit from BD_L therapy.

At the end of Objective 1, we will have developed a mBD_L prototype that can be administered to mice on any MHC background without being rejected. We will also have learned the level of GITRL and the ratio of CXCR5:CCR7 expression that drives therapeutic levels of Treg expansion. We will have also tested the mBD_L prototype in numerous animals models in which disease severity, mBD_L half-life and Treg numbers will be quantitated along the disease course. The information gained will be directly utilized for the construction of a universal human BD_L (hBD_L) therapeutic in Objective B that is optimized for maximal activity.

Objective 2. Generation of a universal off-the-shelf human BD_L (hBD_L) ACT

Currently, ACT are individualized requiring genetic manipulation and in vitro expansion of a patient's own cells prior to administration back to the patient. Our goal is to develop a BD_L-based ACT that could be administered to any patient. We are calling this a "universal off-the-shelf" product. In order to achieve this goal, we will use an Epstein Barr Virus (EBV) transformed B cell to facilitate genetic engineering. We will select one EBV transformed B cell clone that induces high levels of Treg proliferation to move forward in the pipeline. Because we cannot administer transformed BD_L to humans, we will genetically manipulate the clone so that its proliferation can be conditionally shut off. The universal off-the-shelf hBD_L ACT will be in vitro expanded to therapeutic levels prior to rendering it proliferation incompetent and administration to the patient. This strategy allows the usage of the same hBD_L product for a multitude of patients.

The generation of the mBD_L prototype will guide the strategy in human in regards to required expression levels of key BD_L effector molecules. The methods that will be utilized to modulate their expression in hBD_L will be identical to the mouse except they will be modified to the human system. For instance, HLA-E or HLA-G will be expressed in place of Qa1 and Qa2, respectively. Here, we will concentrate on our strategy to generate a safe BD_L-based ACT. This will include a parent cell choice, strategies to control proliferation and suicide genes. In last section, we will provide a humanized animal model to test the hBD_L therapeutic.

A. Generation of the parent hBD_L

Overview. As described above, B cells do not undergo extended homeostatic expansion. Thus, a self-renewing parent cell is required to allow for genetic manipulation. After much consideration, we determined that starting with a tumor cell line was too risky because the genetic causes of its self-renewal would be difficult to determine and control. The primary mechanism utilized to transform human mature B cells is EBV[83]. We have chosen this strategy for two reasons. First, we can directly transform hBD_L with the required cell surface phenotype and regulatory characteristics. Second, the genetics of how EBV transforms B cells and maintains their self-renewal is well understood. Thus we can manipulate the EBV genome to cease B cell proliferation prior to administration to increase its safety.

EBV genetic manipulation. The mechanisms by which EBV drives B cell proliferation are well defined at the genetic level[84-86]. Our strategy is to generate EBV recombinants by introducing LoxP sites into EBV genes of interest that will be deleted upon expression of an inducible Cre in the parent cell line. EBV target genes are EBNA2, EBNA3C and LMP1, which are required for continuous proliferation of lymphoblastoid cells lines[87]. In addition, we will target the viral origin of DNA replication (oriP) which is essential for maintaining the EBV genome in dividing cells[88]. EBV mutant viruses will be generated for each of the four targets. Our goal is to generate a B cell that is immortal that can be redendered replication incompetent. In addition, the EBV mutant will be engineered to be replication defective so that it poses no risk to the patient or general public. Gene selection and EBV genetic manipulation will be accomplished in collaboration with [redacted][89-91].

EBV transformation. Once genetically modified EBV are created, we will FACS purify hBD_L

($CD19^+CD20^+CD23^-IgD^{low/-}$) (**Fig. 3**) from peripheral blood and they will be transformed with each of the five mutant EBV. The BD_L donor will be female to avoid any potential recognition of male antigens by female patients. EBV transformation will be performed in collaboration with [redacted][92]. Single cell clones will be generated and screened by flow cytometry for the BD_L markers IgD , GITRL, CXCR5 and CCR7. In addition, migration to CXCL13 and CCL21 will be measured. Clones expressing the $IgD^{low/-}$ BD_L phenotype and exhibiting the optimal functional ratio of CXCR5 and CCR7 will be screened in vitro for the ability to induce Treg proliferation (see **Fig. 8)**. Treg from the BD_L donor (autologous) will be utilized to avoid allorecognition.

B. Generation of a hBD_L ACT

The hBD_L clone that induces the greatest level of Treg proliferation will be selected for further genetic engineering to generate a hBD_L cellular therapy in five steps:

1. Assessing cessation of cell proliferation
2. Deletion of B2M, CIITA and CD20
3. Introduction of HLA-E or HLA-G
4. Over expression of GITRL
5. Assess chemokine receptor expression and responsiveness
6. Introduction of suicide genes

Step 1. Assessing cessation of cell proliferation. Once the hBD_L lymphoblastoid lines are generated they will be transduced with an episomal vector[93] containing a tamoxifen inducible Cre[94]. Upon administration of tamoxifen the Cre recombinase will be expressed leading to gene deletion of the EBV floxed genes. Deletion efficiency and gene expression will determined by PCR. Proliferation will be monitored by measuring cell proliferation using a variety of methods including counting cells, BrdU incorporation and Ki-67 expression. Cell survival will be monitored using trypan blue and apoptosis levels will determined by activated caspase 3/7 levels by flow cytometry. If deletion of oriP or single EBV genes does not block proliferation, then we will delete EBV genes in combination.

If we cannot find a combination of EBV genes that when deleted block proliferation while maintaining hBD_L viability, we will then examine host genes targeted by EBV that drive B cell proliferation. Target host genes will be selected from a publication from the Gewurz laboratory guided by results from the single EBV mutants in terms of the extent to which cell proliferation was reduced[95]. The same tamoxifen inducible Cre/loxp3 approach will be utilized for gene deletion in the hBD_L. It is likely that we can find a combination of EBV and host gene deletions that will allow blockage of proliferation while preserving cell survival. When we find the best combination of genes to render the hBD_L lymphoblastoid cells replication incompetent while maintaining long-term survival individual clones will be generated and the clone driving the highest level of Treg proliferation will be moved to steps 2-4. Dr. V. Tarakanvova, MCW, an expert on gammaherpesvirus and host defense[96-100], will guide the decision making tree in these studies.

We are aware that our strategy to block EBV-driven cell proliferation is high risk, but if successful would be highly impactful to the EBV field. Nevertheless, an alternative strategy would be to irradiate or mitomycin-c treat to prevent cell proliferation. This may lead to faster turnover, but because the ACT is off-the-shelf additional administrations are feasible. It is also possible that EBV will not infect and transform BD_L. But using our knowledge of BD_L functional mechanisms, we are confident of being able to genetically manipulate any B cell to acquire BD_L regulatory activity.

Steps 2-4. We estimate that it will take 2-4 years to accomplish step 1. In that same time frame, we will begin steps 2-4 using the same clone chosen for step 1. Steps 2-4 will be carried out similarly to those described for mBD_L except that guide RNAs targeting the human *B2M* and *CIITA* genes will be utilized, HLA-E or HLA-G will be expressed in place of Qa1 and Qa2, respectively, and the human GITRL gene will be utilized. As outlined above for mBD_L, in humans, we envision enhancing engraftment of hBD_L by B cell depletion with anti-CD20. Because residual antibody would lead to depletion of hBD_L, we will knock out CD20 using CRIPR/Cas9. An added benefit is that anti-CD20 B cell depleting therapy could be utilized in patients to eliminate pathogenic B cells, while retaining regulatory hBD_L. We will map the insertion sites of the lentiviral vectors by sequencing and chose those that lie in and around genes not expressed in B cells or known oncogenes to move forward in the pipeline.

Step 5. Assess hBD_L chemokine receptor expression and responsiveness. The advantage of the EBV transformation approach is that the parent cell will be a BD_L. However, the many steps of genetic manipulation may alter its native functions. Thus after each round of genetic manipulation, we will obtain clones and test them for expression levels of CXCR5 and CCL7 and function by measuring migration to CXCL13 and CCL21. If we find that they are altered, we will utilize the same strategy as outlined for the mBD_L to alter the levels of CXCR5 and CCL7 so that optimal migration to their ligands is achieved.

Step 6. Introduction of suicide genes. A caveat to the Cre/loxp3 deletion system is that the deletion efficiency will not be 100%. Thus, a strategy needs to be included to induce death in cells that did not undergo deletion and remain proliferative. Our strategy is to introduce suicide genes under the control of the Ki-67 promotor. Ki-67 expression is cell cycle dependent and thus once proliferation is blocked its gene transcription will rapidly cease[101]. There are multiple choices for suicide genes that could be utilized[102]. Here, we outline our top two choices selected for sensitivity and safety in humans. They were also chosen in consultation with [redacted])[103], because they will not lead to bystander cell death. By utilizing the Ki-67 promotor, we can introduce multiple suicide genes to build in redundancy thereby reducing the risk of hBD_L becoming a "leukemia-like" syndrome.

Herpes simplex virus-1 (HSV) Thymidine Kinase (TK)/Ganiciclovir (GCV). TK initiates the phosphorylation of deoxythymidine to deoxythymidine monophosphate, which is then turned into deoxythymidine triphosphate by host cell endogenous kinases[104]. This system can be exploited to drive cell death because TK converts GCV to its toxic metabolite GCV-GP[105]. The incorporation of GCV-GP into DNA leads to single-strand breaks and inhibition of cellular DNA polymerase leading to apoptosis[106]. HSVTK has a 1000-fold higher affinity to GCV than the endogenous TK; therefore, when present, HSVTK predominately initiates the first step of GCV phosphorylation[107]. hBD_L will be lentiviral transduced to express HSVTK using a strong promoter. Numerous studies have outlined strategies to increase the activity of HSVTK such that the dose of GCV required can be reduced thereby decreasing its toxic effects[102]. One of these mutations will be incorporated into the HSVTK construct. hBD_L cells will be treated with GCV following tamoxifen treatment and cell proliferation and survival will be determined as above. Should a proliferating hBD_L escape and expand in a patient, GCV could be administered since it is an FDA approved drug.

Inducible Caspase 9. Caspase 9 plays a role late in apoptosis and its forced dimerization with the synthetic drug AP1903 led to death of 99% of T cells selected for high transgene expression in vitro and in vivo[108]. A variant human caspase 9 lacking the CARD and both FKBP domains (iCasp9) prevented multimerization with the added benefit of higher expression and increased sensitivity to apoptosis[108]. AP1903 is a bioinert small molecule deemed safe in humans[109]. In a clinical trial iCasp9 was introduced into donor T cells given to enhance immune reconstitution following BMT and AP1903 was administered to patients who developed GVHD leading to loss of >90% of the modified T cells within 30 minutes and ended GVHD without recurrence[110]. Our strategy of immortalization combined with inducible death has been validated in a human mesenchymal stromal cell line[111]. hBD_L will be transduced to express iCasp9 and tested as for HSVTK.

One concern of introducing HSVTK into hBD_L is that it will be seen as foreign by the immune system leading to their clearance. However, because the only MHC gene hBD_L will express is HLA-E or -G antigen presentation will be limited making it very unlikely that an immune response against HSVTK would occur. Nevertheless, we will utilize SVMHC to identify HSVTK peptides predicted to bind HLA-E or HLA-G[112]. The predicted peptides will be generated and tested for binding to HLA-E or -G. If potential immunogenic peptides are identified, we will mutate either a TCR or MHC contact residue to disrupt binding. The variant HSVTK will be tested for function. iCasp9 is human so it should not trigger an immune response. If a rogue hBD_L escapes both the HSVTK and iCasp9 approaches they could still be controlled by deletion with anti-CD19, which is being developed and tested in clinical trials (ClinicalTrials.gov).

C. Testing hBD_L in animal models. The final stage of developing a hBD_L therapeutic will be testing in animal models. Testing human cellular products in mice is challenging, but has been advanced with the generation of humanized mice[113,114]. The model we will utilize are NSG mice from Jackson Laboratories. NSG mice are NOD mice carrying the scid mutation, which is a loss-of-function mutation of the *Prkdc* gene that prevents T and B cell development. NSG mice are also null for the IL-2Rγ chain, which diminishes signaling for IL-2, IL-4, IL-7,

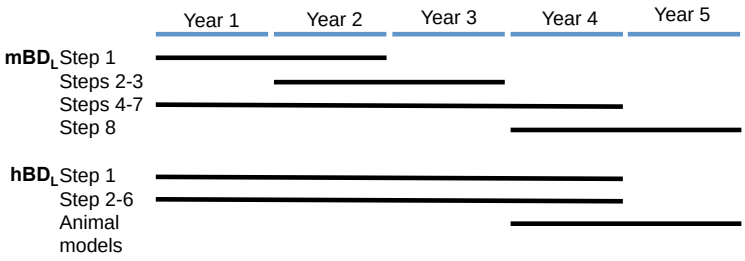
IL-9, IL-15 and IL-21. The loss of IL-15 prevents NK cell development. We will utilize the NSG-SGM3 mouse that expresses human IL-3, GM-CSF and SCF allowing for stable engraftment of myeloid lineages and Treg populations[115]. The mice will receive human $CD34^+$ hematopoietic stem cell transplants to reconstitute the human immune system. The transplanted mice will be generated in the Dittel laboratory in collaboration with [redacted], who has extensive experience generating humanized mice[116]. Following immune reconstitution endogenous B cells will be depleted with anti-human CD20 and hBD_L ($CD20^-$) (irradiated or mitomycin-c treated) will be adoptively transferred and their longevity and turnover rate will monitored for up to one year. We will also track Treg numbers. We are particularly interested in whether any hBD_L escapees emerge and expand in the mice. To specifically test the effectiveness of our suicide genes, replication defective hBD_L will be spiked with 1-1000 replication competent hBD_L and their cell numbers will be tracked in the peripheral blood weekly. We anticipate that the replication competent hBD_L will expand and when they reach various levels, we will treat the mice with GCV and iCasp9 alone and in combination. Loss of hBD_L and Treg numbers will be quantitated at various timepoints in multiple lymphoid organs. A cohort of mice will be taken off drug treatment and watched for reemergence of proliferating hBD_L. This cycle will be repeated to determine whether long-term control of hBD_L numbers can be achieved.

At the end of this objective, our goal is to have a hBD_L that would be ready for additional safety testing required for a phase I safety trial. To achieve that goal, once each step is completed it will be repeated under GMP conditions. The BRI has a working GMP facility (see facilities). Because of the link between BD_L and Treg numbers, our universal hBD_L therapy would most benefit those autoimmune diseases that are responsive to anti-CD20 depletion therapy. These include rheumatoid arthritis, systemic vasculitis, pemphigus vulgaris, autoimmune hemolytic anemia, myasthenia gravis, multiple sclerosis and others[75,117].

Innovation. The strategy of generating a universal off-the-shelf ACT that cannot be recognized by the immune system is a first-of-its-kind drug that will revolutionize the treatment of autoimmunity. Currently, there are no ACT for autoimmunity and none that utilize B cells to reset the homeostatic balance of the immune system towards an anti-inflammatory state. In addition, hBD_L ACT will be refractory to anti-CD20 depletion allowing combination therapies that deplete pathogenic B cells while retaining regulatory B cells. Our hBD_L ACT strategy is based on our recent discovery of BD_L, which are a new B cell subset that play a critical role in immune tolerance by maintaining Treg homeostasis. Discovery of a new immune cell subset is a major accomplishment, which places us in the unique position to translate our findings to humans for the treatment of inflammatory diseases. To accomplish our goal, we devised a strategy to immortalize BD_L with EBV and then shut down their proliferation by deleting viral genes required to sustain B cell proliferation. This is a novel approach that has the potential to advance our study of B cell biology and EBV-derived lymphoma/leukemia.

Appropriateness for the Transformative Award. A hBD_L ACT is appropriate for the transformative award because it meets the objective of developing a highly innovative therapy. Our use of hBD_L is substantially different from T cell-mediated ACT in that it can treat a broad spectrum of inflammatory disorders with a single product. Autoimmunity and other inflammatory diseases are often chronic and thus are a major burden on healthcare costs. A universal off-the-shelf ACT that dampens inflammation will not only lower the cost of healthcare but the well-being of the patient will dramatically increase. This will have the added economic benefit of patients having a higher quality of life and remaining in the workforce requiring less medical assistance. We are also developing a novel method to control EBV transformed lymphoblast proliferation, which has the potential to lead to new therapies targeting EBV-derived B cell lymphoma/leukemia.

Timeline. The generation of mBD_L and hBD_L will occur simultaneously and take all five years. An estimated timeline is shown. Briefly, selection of the parent mBD_L (step 1) is estimated to take 2 years with activity screening occurring in years 2-3 (steps 2-3). EBV transformation of the hBD_L will take place in year 1 (part of step 1) with EBV genetic manipulation occurring in years 1-4 (step 1). Generation of the genetic strategies for both mBD_L (steps 4-7) and hBD_L (steps 2-6) will occur in years 1-4. Testing in animals for both the mBD_L (step 8) and hBD_L will occur in years 4-5.



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