CD38 Correlates with an Immunosuppressive Treg Phenotype in Lupus-Prone Mice

Jocelyn C. Pérez-Lara 1,*, Enrique Espinosa 2, Leopoldo Santos-Argumedo 3, Héctor Romero-Ramírez 3, Gabriela López-Herrera 4, Fabio García-García 5, Claudia Sandoval-Montes 6, Vianney Ortiz-Navarrete 3, and Juan C. Rodríguez-Alba 1,8

Abstract: CD38 is a transmembrane glycoprotein expressed by T-cells. It has been reported that patients with systemic lupus erythematosus (SLE) showed increased CD38+CD25+ T-cells correlating with immune activation and clinical signs. Contrariwise, CD38 deficiency in murine models has shown enhanced autoimmunity development. Recent studies have suggested that CD38+ regulatory T-cells are more suppressive than CD38− regulatory T-cells. Thus, we have suggested that CD38 overexpression in SLE patients could play a role in regulating immune activation cells instead of enhancing it. This study found a correlation between CD38 with FoxP3 expression and regulatory T-cells correlating with immune activation and proliferation after stimulation through the T-cell receptor (TCR). Finally, we demonstrated an increased ratio of IFN-γ+T-cells from lupus-prone mice compared with WT. Altogether, our data suggest that CD38 represents an element in maintaining activated and proliferative Treg cells. Consequently, CD38 could have a crucial role in immune tolerance, preventing SLE development through Treg cells.

Keywords: CD38; regulatory T-cells; systemic lupus erythematosus; immunosuppressive; lupus-prone mice; IFN-γ; IL-10

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic, multiorgan, systemic autoimmune disease that affects nearly every tissue and organ system [1]. As with other autoimmune diseases, SLE more frequently affects women than men, with female to male ratios exceeding 9:1 [2]. The etiological mechanism of SLE remains unclear. However, genetic,
hormonal, and environmental factors and a loss of tolerance have been associated with disease development [3,4]. Preliminary studies of genetic factors have demonstrated that European–American and Icelandic populations have an SLE susceptibility locus on the human region 4p15 [5,6]. Located within the 4p15 region is the gene encoding CD38 [7], a multifunctional transmembrane glycoprotein that has both enzymatic and receptor properties [8,9]. As an enzyme, CD38 can convert nicotinamide adenine dinucleotide (NAD+) into cyclic adenosine diphosphate ribose (cADPR) [10]. Otherwise, activation of CD38 receptor activity induces several cell responses such as activation, migration, proliferation, differentiation, and apoptosis in different immune cell types [11–14]. Previous studies have shown increased levels of CD38 expressing T and B cells in SLE patients compared to healthy controls [15–18]. Remarkably, Pavón et al. reported higher levels of CD38 expression in CD25+ T-cells from SLE patients than in healthy controls [19], suggesting that CD38 plays a role in preventing autoimmunity. Several studies have found that CD38 absence in murine autoimmune models enhances illness severity [20,21]. We have demonstrated that CD38 deficiency in a non-autoimmune-susceptible strain (CD38−/−) developed some autoimmune characteristics at old age [22], suggesting that CD38 could play a role in maintaining immune homeostasis. Furthermore, CD38 was upregulated in regulatory B cells (Bregs), and CD38 cross-linking with an agonistic antibody increased the frequency of Breg cells and IL-10 production [22]. Altogether, it seems that the CD38 receptor can play an essential role in preventing autoimmunity mechanisms, mainly through the regulation of the immune system.

Regulatory T-cells (Treg cells) play a crucial role in regulating the immune response, maintaining peripheral tolerance, and controlling the development of several autoimmune diseases, such as SLE [23]. Treg cells are identified by CD25 and FoxP3 expression in CD4+ T-cells from mice, while CD4+CD25hiCD127low T-cells have been widely accepted to identify Treg cells in humans [24,25]. Additional markers have been described to select functional Treg cells, highlighting immunosuppressive molecules such as CTLA-4, IL-10, CD69, and PD-1 [26–29]. A further vital mechanism to maintain immune tolerance is Treg proliferation, which is required to expand these suppressive cells and avoid autoimmune diseases [30].

In the last few years, an increasing number of studies in patients with multiple myeloma have found that CD38+ Treg cells are more suppressive than CD38− Treg cells and antagonist antibodies can block their inhibitory activity [31,32]. Despite this, the role of CD38 in SLE disease has not yet been established. Therefore, we hypothesized that CD38 expression in T-cells could play a role in immune regulation instead of immune activation. To evaluate this, we correlated CD38 expression in T-cells with FoxP3 expression and immunosuppressive markers in lupus-prone mice. Furthermore, we assessed immunosuppressive molecules and proliferative capability in Treg cells from CD38−/− mice. Finally, we defined that CD38 deficiency promoted a dysregulated IFN-γ/IL-10 cytokine secretion when splenocytes were stimulated with anti-CD3/CD28 + rhIL-2. We found that CD38 expression is essential for maintaining the immunoregulatory phenotype and expanding Treg cells from lupus-prone mice.

2. Results

2.1. CD38 Expression Positively Correlates with FoxP3 Expression

To assess the regulatory role of CD38 in T-cells from a lupus-prone mouse (B6.MRL-Fas+/+), we examined FoxP3 expression in three different subsets of CD3+CD4+CD25− and CD3+CD4+CD25+ T-cells, gated according to CD38 expression. As shown in Figure 1A, we identified CD38low, CD38mid, and CD38high cells within CD4+CD25− and CD4+CD25+ T-cell subsets from WT and B6.MRL-Fas+/+ mice. We then analyzed the frequency of FoxP3 expressing cells in each subset (Figure 1B). As shown in Figure 1B, we noted an increased amount of Foxp3+ cells when CD38 increased its expression in CD25+ and CD25− T-cells.
cells from WT and B6.MRL-Faslpr/J mice. Thus, we assessed the relationship between the surface expression of CD38 and the transcription factor, FoxP3. We plotted the mean fluorescence intensity (MFI) of CD38low, CD38mid, and CD38high against the number of cells positive for FoxP3 from CD25+ and CD25− cells in WT and B6.MRL-Faslpr/J mice. Using Spearman correlation analysis, we displayed a positive correlation between CD38 and frequency of FoxP3 cells among CD4+CD25− from WT (rS = 0.87, p < 0.01) and B6.MRL-Faslpr/J mice (rS = 0.94, p < 0.05) (Figure 1C). In the same way, CD4+CD25+ cells showed a positive correlation between CD38 and Foxp3 cells in WT (rS = 0.73, p < 0.01) and B6.MRL-Faslpr/J mice (rS = 0.90, p < 0.05) (Figure 1C). Additionally, correlation plots showed higher CD38 expression in CD25+ and CD25− cells from B6.MRL-Faslpr/J than WT mice. These results suggest that CD38 expression may identify subsets with regulatory characteristics.

Figure 1. The level of CD38 correlates with FoxP3 frequency in T CD25+ and CD25− T-cells. Splenocytes were stained for cell surface expression of CD3, CD4, CD25, and CD38. The stained cells were fixed and stained intracellularly for FoxP3. (A) Splenocytes from wild type (WT) and B6.MRL-Faslpr/J were gated on CD3+CD4+ cells and analyzed for CD25 expression. Then, CD3+CD4+CD25+ or CD25− cells were gated on CD38low, CD38mid, or CD38high, according to CD38 expression; (B) counter plots showing FoxP3+ cells frequency in CD38low, CD38mid, or CD38high subsets among CD25+ and CD25− T-cells from WT and B6.MRL-Faslpr/J; (C) correlation plots comparing MFI of CD38 to FoxP3+ cells within either CD4+CD25− T-cells (top) or CD4+CD25+ T-cells (bottom). Symbols represent T-cells from WT (n = 4) (open symbols) and B6.MRL-Faslpr/J (n = 6) (filled symbols). The inset lines are the product of linear regression analysis. Correlations were calculated by the Spearman method.
2.2. CD38 Expression Positively Correlates with Immunosuppressive Molecules

To understand the possible regulatory relevance of CD38 expression in CD4+CD25+ and CD4+CD25− T-cells, we assessed by flow cytometry the MFI of CD69, IL-10, CTLA-4, and PD-1 from CD38low, CD38mid, and CD38high subsets from WT (Figure 2A,B) and B6.MRL-FasΔFas−/− (Figure 2C,D). Then, we developed a Spearman correlation matrix including MFI values from each CD38 subset and immunosuppressive molecule MFI values. CD4+CD25+ T-cells from WT mice showed a positive correlation between CD38 and PD-1 ($r_S = 0.95$, $p < 0.05$), CTLA-4 ($r_S = 0.95$, $p < 0.05$), and IL-10 ($r_S = 0.6$, $p < 0.05$) (Figure 2A). Otherwise, CD38 expression in CD4+CD25− T-cells were highly correlated with only CD69 protein ($r_S = 0.97$, $p < 0.05$) (Figure 2B). Regarding B6.MRL-FasΔFas−/− mice, levels of CD38 expression in CD4+CD25+ cells exhibited a strong positive correlation with PD-1 ($r_S = 0.81$, $p < 0.05$) and CD69 ($r_S = 0.84$, $p < 0.05$) (Figure 2C), whereas that of CD4+CD25− cells showed CD38 correlation with PD-1 ($r_S = 0.85$, $p < 0.05$), CTLA-4 ($r_S = 0.9$, $p < 0.05$), and CD69 ($r_S = 0.94$, $p < 0.05$) (Figure 2D). Together, these data proposed that CD38 expression is consistent with an activated immunosuppressive phenotype.

Figure 2. CD38 correlates with immunosuppressive molecules. Correlation matrix and histograms of PD-1, CTLA-4, IL-10, and CD69 expression within CD38low (dashed line), CD38mid (black line), and CD38high (heavy line) subsets in (A) WT CD4+CD25+ T-cells ($n = 6$); (B) WT CD4+CD25− cells ($n = 6$); (C) B6.MRL-FasΔFas−/− CD4+CD25+ T-cells ($n = 6$); and (D) B6.MRL-FasΔFas−/− CD4+CD25− T-cells ($n = 6$). Darker squares into correlation matrices point out a correlation close to 1. Shaded squares indicate correlations with a $p < 0.005$, while blank squares show non-significative correlations.

2.3. Reduced CD38+ Treg Proportion in B6.MRL-FasΔFas−/− Mice

Because CD38 correlates with immunosuppressive molecules in T-cells from WT and B6, MR2-FasΔFas−/− mice, and it could select subsets with suppressive relevance, we wondered if CD38+ Treg cells frequency might be impaired in B6.MRL-FasΔFas−/− mice. First,
we identified two main subsets of Treg cells, CD38$^+$ and CD38$^-$ in WT and B6.MRL-Fas$^{lp}$/J mice (Figure 3A). Interestingly, the frequency of splenic CD38$^+$ Treg cells was significantly decreased from B6.MRL-Fas$^{lp}$/J in comparison with WT mice ($T_{15} = 5.3, p < 0.0001$) (Figure 3A,C). Consistently, B6.MRL-Fas$^{lp}$/J mice showed a decreased proportion of total Treg cells ($\chi^2(2) = 17.48, p < 0.01$) (Figure 3A,B). To figure out CD38 relevance in Treg maintenance, we assessed splenic Treg frequency in CD38$^-$/- mice. Compared with WT mice, CD38$^-$/- mice showed a lower frequency of Treg cells ($\chi^2(2) = 17.48, p < 0.001$), and non-significative differences were found against B6.MRL-Fas$^{lp}$/J model (Figure 3B). These results suggested that the development of Treg seems to be affected by the lack of CD38.

Figure 3. Reduced CD38$^+$ Treg levels in B6.MRL-Fas$^{lp}$/J mice. (A) Representative dot plots of the frequency of Treg cells in splenocytes from WT, B6.MRL-Fas$^{lp}$/J and CD38$^-$/-. The dot plots’ right is representative contour plots showing CD38 expression gated on splenic Treg cells from WT ($n = 9$), B6.MRL-Fas$^{lp}$/J ($n = 8$), and CD38$^-$/- mice ($n = 8$); (B) scatter dot plot shows mean ± SD of Treg frequency from WT, B6.MRL-Fas$^{lp}$/J and CD38$^-$/- mice. Statistical analysis was performed using one-way ANOVA. Differences between groups were indicated by Tukey post hoc test, where * $p < 0.001$, ** $p < 0.0001$; (C) scatter dot plot shows mean ± SD of CD38$^+$ Treg frequency from WT and B6. MRL-Fas$^{lp}$/J mice. Statistical analysis was performed using Student t-test, where ** $p < 0.0001$.

A previous study in CD38$^-$/- mice has revealed some autoimmune disorders in aged mice [22]. Therefore, we asked whether CD38 could be essential to maintain a regulatory phenotype. Therefore, we measured the expression of immunosuppressive molecules in total splenic Treg cells from CD38$^-$/- mice, and we compared their expression with CD38$^+$ and CD38$^-$ Treg cells from WT and B6.MRL-Fas$^{lp}$/J mice. In the first instance, CD38$^+$ Treg cells from WT and B6.MRL-Fas$^{lp}$/J expressed higher levels of CD69 (Figure 4A), IL-10 (Figure 4B), and PD-1 (Figure 4D) than CD38$^-$ Treg cells, while CD38$^+$ Treg overexpressed
CTLA-4 from B6.MRL-Fas<sup>lpr</sup>/J mice (Figure 4C). Interestingly, when we assessed MFI of CD69, IL-10, CTLA-4, and PD-1 in total splenic Treg from CD38−/− mice, we noted a relevant reduction in their expression compared with CD38<sup>+</sup> Treg from WT and B6.MRL-Fas<sup>lpr</sup>/J mice (Figure 4A–D). Together, results showed that CD38 expression is associated and required to maintain critical suppressive markers.

**Figure 4.** Treg from CD38−/− showed a reduction in immunosuppressive molecules. Histograms and bar charts represent the expression of (A) CD69, (B) IL-10, (C) CTLA-4, and (D) PD-1 among CD38− and CD38<sup>+</sup> Treg cells from WT (<i>n</i> = 4) and B6.MRL-Fas<sup>lpr</sup>/J (<i>n</i> = 4) mice or splenic Treg cells from CD38−/− (<i>n</i> = 4) mice. Bar charts show mean ± SD. Statistical analysis was performed using two-way ANOVA followed by Tukey test, where ***<i>p</i> < 0.0001, **<i>p</i> < 0.001, and *<i>p</i> < 0.05.

### 2.4. Reduced Treg Expansion and IL-10 Secretion in Stimulated CD38−/− Splenocytes

Given that TCR signals play a crucial role in Treg cell maintenance and proliferation [33,34], we examined whether the lack of CD38 could impair the proliferative ability of Treg cells stimulated via CD3. Total splenocytes from WT and CD38−/− mice were left unstimulated or stimulated with different concentrations of anti-CD3 for 72 h, as described in materials and methods. After cell surface staining for CD4 and CD25, cultured cells were intracellularly stained for Foxp3. As depicted in Figure 5A,B, Treg cells from CD38−/− mice showed significantly reduced frequency than WT mice specially at 0.01 (T<sub>4</sub> = 16.07, <i>p</i> < 0.0001) and 0.1 µg/mL (T<sub>4</sub> = 20.7, <i>p</i> < 0.0001) of anti-CD3.

Splenocytes stimulated with anti-CD3/CD28 + rhIL2 or mitogens such as phorbol myristate acetate plus ionomycin (PMA/IONO) promote the release of pro-inflammatory and anti-inflammatory cytokines such as IFN-γ and IL-10, respectively [35,36]. A good balance between the pro-inflammatory IFN-γ and anti-inflammatory IL-10 is an indicator of reciprocal immune regulation [37,38]. Therefore, to understand the role of CD38 in this phenomenon, we analyzed the ability of the expanded splenocytes from CD38−/− mice to produce IFN-γ that might either enhance or abrogate Treg function. Analysis of culture supernatants of CD38−/− splenocytes stimulated with anti-CD3/CD28 + rhIL2 and PMA/IONO showed a reduced concentration of IFN-γ and IL-10 compared to supernatants
of WT splenocytes (Figure 5C,D). In addition, the ratio of IFN-γ/IL-10 was detected to be significantly higher in supernatants from CD38−/− stimulated splenocytes, compared with WT splenocytes (Figure 5E).

Figure 5. Reduced Treg expansion and IL-10 secretion in stimulated CD38−/− splenocytes. Splenocytes from WT (n = 3) and CD38−/− (n = 3) were left unstimulated or stimulated with indicated concentrations of anti-CD3 for 72 h. Cells were stained with anti-CD4 and anti-CD25, followed by intracellular staining for Foxp3. (A) Representative dot plots of Treg gated according to CD25 and FoxP3 expression. Numbers in the oval indicate the percentage of Treg cells after stimulation; (B) percentage (mean ± SEM) of Treg cells obtained from the culture; (C) concentrations (mean ± SEM) of IFN-γ and (D) IL-10 release in splenocyte-culture supernatant upon in vitro stimulation with anti-CD3/CD28 + rhIL-2 for 72 h; (E) IFN-γ/IL-10 ratio. Statistical analysis was performed using two-way ANOVA followed by Tukey test, where **p < 0.001, and *p < 0.05.

3. Discussion

An unprecedented number of studies performed in SLE patients have demonstrated that CD38 is widely expressed in T-cells, showing that CD25+CD38+ T-cells are increased regarding healthy controls [15–19]. It has been proposed that the increase in CD25+CD38+ T-cells in SLE disease could be indicative of persistent T-cell activation [19]. However, the contribution of CD38 to the pathology of SLE remains unclear. Since CD38 is a transmembrane glycoprotein widely expressed in several immune cells, its function during SLE development might involve different pathways and effector functions according to the cell type where it is expressed.

Previous reports have described that CD38 expression might identify CD4+ T-cell subsets with immunomodulatory properties [39]; thus, we hypothesize CD38 expression in T-cells from SLE patients can mediate disease development through regulatory activity. To assess it, we used B6.MRL-Fas(+/−)/J lupus murine model, which is widely used to understand autoimmune disease’s physiological and pathological processes [40,41]. Thus, first, we analyzed and compared regulatory phenotype through FoxP3 expression in different subsets of CD4+ T-cells divided according to CD25 and CD38 expression in WT and B6.MRL-Fas(+/−)/J mice. We identified three different levels of CD38 expression (CD38high, CD38mid, CD38low) within CD4+CD25+ T-cells and CD4+CD25− T-cells. In this
We have suggested that a reduced CD38, mainly produced by CD103, acts as an inducer of Treg response in CD4 T-cells between CD38 and PD-1, CTLA-4, and IL-10, while in B6.MRL-Fas promoter and activation of FOXP3 expression [44,45]. An environment enriched with CD38-/− could revert or prevent SLE development in B6.MRL-Fas+/−lpr/J mice, as previous results were

In this work, we confirmed a Treg deficiency in CD38−/− mice, suggesting that CD38 is relevant to maintain a suppressive phenotype. Previously, CD38−/− Treg cells have been defined as a highly suppressive subset in patients with multiple myeloma [31,32] and have shown an increased ability to inhibit immune response [42]. We have suggested that a reduced CD38−/− Treg could indicate Treg loss with suppressive function in B6.MRL-Fas+/−lpr/J mice. Thus, we proposed that CD38 expression in Treg might identify functional Treg cells, highlighting that a higher proportion of CD38−/− Treg cells could revert or prevent SLE development in B6.MRL-Fas+/−lpr/J mice.

CD38 is a transmembrane glycoprotein with ectoenzyme and receptor activity, such as ectoenzyme CD38, the principal NAD+ consumer and regulator in several conditions [54]. Previous reports have established that NAD+ conversion through CD38 may generate adenosine (ADO), which has been associated with inducing FoxP3 expression and promote CTLA-4 expression, significantly increasing immunoregulatory activity [55,56]. Furthermore, it has been shown that CD38 absence in murine models with autoimmunity enhances the development of the disease. More recently, our laboratory found that only CD38 deficiency in a WT background induced an autoimmune phenotype such as SLE [22]. In this work, we confirmed a Treg deficiency in CD38−/− mice, as previous results were.
reported [42,57]. Explanation to this loss of Treg cells in CD38−/− mice include the role of CD38 in removing NAD+ and preventing its binding to the mono-ADP-ribosyltransferase ART2, an enzyme that ribosylates P2X7 and induces apoptosis of CD4+CD25+ cells [57–59].

We also reported that Treg cells from CD38−/− mice showed an impaired expansion ability when stimulated via CD3. Even though there is no evidence of CD38 signaling in Treg, several studies have proposed that CD38 signaling in T-cells is initiated within a subset of membrane rafts [60], activating a transduction pathway that includes ζ-associated protein-70, CD3ε, phospholipase C-γ, Raf-1/mitogen-activated protein kinase, and calcium mobilization [61–63]. Additionally, CD38 signaling requires a T-cell receptor (TCR)/CD3 receptor complex, suggesting a functional association with TCR signal transduction machinery [61–63]. Remarkably, we proposed that CD38 also participates in the TCR signaling pathway to maintain Treg cells.

It is known that induction of IFN-γ often occurs together with anti-inflammatory cytokines such as IL-10 as a mechanism to reduce the uncontrolled production of inflammatory cytokines [37,38]. Here, we found the ratio IFN-γ/IL-10 decreased in CD38−/− mice than WT. These findings are in contradiction with data previously reported by Burlock and cols., which mentioned that CD38 deficiency increased IL-10 producing B cells and reduced IFN-α production in the peritoneal cavity [64] and with Domínguez and cols., who found no differences in IL-10-producing B cells between CD38−/− and WT mice [22]. In this work, we are evaluating secretion of IL-10 from splenocytes stimulated with anti-CD3 and PMA/IONO stimulus for 72 h, identifying IL-10 released after the proliferation of T-cells or total splenocytes, respectively. In contrast, they identified mainly intracellular IL-10 in B10 cells by shorter periods of stimulation with LPS. Otherwise, CD38 blocking with Isatuximab in Treg cells from Myeloma Multiple patients has demonstrated a reduced IL-10 expression and a higher amount of IFN-γ expression in CD8+ T-cells [31]. However, mechanisms of IL-10 secretion are different in each cellular type; thus, we have to perform additional experiments to elucidate if Treg from CD38−/− loss ability to produce IL-10, an important mechanism to maintain tolerance and avoid the development of autoimmune disease in CD38−/− mice of advanced age.

As a whole, our data demonstrate that CD38 correlates with FoxP3 expression and immunosuppressive markers in a murine model of SLE. Thus, we proposed that CD38 expression is vital to maintain Treg cells homeostasis and to prevent autoimmune disease. Future work will focus on characterizing the absence of CD38 in B6.MRL-Fas<sup>bp</sup>+/J mice and its effect on the immune regulation to clarify controversial data supporting both beneficial and pathogenic roles for CD38 in SLE disease.

Furthermore, additional studies must be performed to understand the interaction between CD38 receptor and TCR to induce functional Treg cells. Nevertheless, the present findings might help to suggest new therapeutic strategies in SLE.

4. Materials and Methods

4.1. Mice

C57BL/6 wild-type (WT), C57BL/6 CD38−/− (CD38−/−), and C57BL/6-MRL-Fas<sup>bp</sup>+/J (B6.MRL-Fas<sup>bp</sup>+/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). According to national regulations, the mice were bred by strain to obtain littermates in the Universidad Veracruzana animal facility. Male mice between 8 and 12 weeks old were used for experiments. All mice were maintained in specific pathogen-free conditions with a 1:1 light-dark cycle. Food and water were provided ad libitum.

Institutional Animal Care approved all procedures for animals (No. 2018-0004). In addition, the authors adhere to the guidelines established by Mexico (Norma Oficial Mexicana NOM-062-ZOO-1999).

4.2. Isolation of Splenocytes

Mice were euthanized by cervical dislocation, and their spleens were excised. Splenocytes were dissociated from the connective tissue capsule by gently pressing the organ
through a 200-mesh sterile metal sieve. Erythrocytes were depleted with 0.85% ammonium chloride solution. Cells were washed once with phosphate-buffered saline (PBS 1×) and resuspended in PBS 1× (Corning, Manassas, VA, USA).

4.3. Flow Cytometry

For surface staining, cells were stained with anti-CD3 PerCP-Cy5.5, anti-CD4 APC, anti-CD25 APC-Cy7, anti-CD69 Pe-Cy7, anti-PD-1 BV421, and anti-CD38 Alexa Fluor 488 (all from BD Biosciences, San Diego, CA, USA). Cells were then incubated for 15 minutes under room temperature and were fixed with 1% formaldehyde for 15 minutes. For intracellular staining, cells were permeabilized (Cytofix/CytopermTM Fixation/Permeabilization Solution Kit, BD Biosciences) for 40 minutes and stained with anti-FoxP3 Alexa Fluor 488, anti-CTLA-4 BV605, and anti-IL-10 APC. All the samples were analyzed using an LSRFortessa flow cytometer (Becton-Dickinson, San Jose, CA, USA) and analyzed by FlowJo software (FlowJo, Tree Star, Ashland, OR, USA).

4.4. Expansion Treg Assay

Cells (1 × 10^6 cells/mL) were set up in 24-well tissue culture plate wells (Corning, Manassas, VA, USA) in a RPMI-1640 medium containing 10% fetal bovine serum, 100 µmol/L non-essential amino acids (Sigma Chemicals, St. Louis, MO, USA), 1 mmol/L sodium pyruvate (Gibco), 0.2 mg/mL penicillin (Gibco), 0.5 mg/mL streptomycin (Gibco), HEPES 10 mmol/L, and 5.5 × 10−5 mol/L β-mercaptoethanol (Gibco). Afterward, cells were left unstimulated or stimulated with an anti-CD3ε Ab (BD Biosciences, San Diego, CA, USA) at the indicated concentrations. The plates were incubated at 37 °C in the presence of 5% CO2. After incubation, the cells were harvested and washed with PBS. Finally, they were stained with anti-CD4 APC, anti-CD25 APC-Cy7, and anti-FoxP3 PE, as we described above.

4.5. IFN-γ and IL-10 Detection

Splenocytes from WT and CD38−/− mice were left unstimulated or stimulated with 1 µg/mL of anti-CD3ε Ab (BD Biosciences, San Diego, CA, USA), 1 µg/mL of anti-CD28 Ab (BD Biosciences, San Diego, CA, USA), and 200U rhIL-2 (Sigma Aldrich) or Phorbol Myristate Acetate (50 ng/mL) plus Ionomycin (500 ng/mL) for 72 h. According to the manufacturer’s protocol, supernatant concentrations of IFN-γ and IL-10 were evaluated by Mouse ELISA MAXTM DELUXE Set (Biolegend, San Diego, CA, USA).

4.6. Statistical Analysis

Shapiro–Wilk tests were used to test the normality of the data distributions. Correlation tests were performed between CD38 and FoxP3, CTLA-4, CD69, PD1, and IL-10 using the non-parametric Spearman test, where an “r” between 0.8 and 1 indicated a high positive correlation; from 0.4 to 0.79, a mean positive correlation, and from 0.01 to 0.39, a low positive correlation was considered. The results were statistically significant when the value of p < 0.05. Treg and Treg CD38+ frequency was performed using the non-parametric Kruskal–Wallis and t-Student tests, respectively, where a p < 0.05 was considered statistically significant. Finally, the cell culture results were evaluated by a two-way analysis of variance (ANOVA). Tukey post hoc test was used for multiple comparisons, as indicated. Statistical significance was determined with p < 0.05. All data were analyzed using the statistical program RStudio 1.3.1093, © 2009–2020 (RStudio, PBC Inc., Boston, MA, USA).

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