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Differences in T regulatory cells between mouse strains frequently used in immunological research
Treg cell quantities and subpopulations in NOD, B6 and BALB/c mice

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ABSTRACT

Foxp3$^+$ Regulatory T cells (Tregs) are essential for the maintenance of tolerance to self. Therefore, it is expected that lower numbers and/or less than optimal function could impact on the functioning of the immune system, and thereby contributing to the development of autoimmune diseases. In the present report, by comparing Tregs from most frequently used mouse strains in immunological research (C57BL/6 (B6), BALB/c and NOD), we provide evidence showing that the NOD mouse strain, highly predisposed to develop autoimmune responses, exhibit a generalized decreased in Tregs counts with enhanced proportions of CD44^{hi}CD62L^{low} Tregs when compared with BALB/c mice. No major differences were observed in Helios$^+$ or Helios- Tregs between strains. The expression of CXCR3, CCR5 and CCR6 on Tregs from all strains showed minor proportions of CXCR3$^+$ and CCR5$^+$ cells in NOD Tregs. Naïve CD4$^+$CD25- T cells from NOD mice also showed decreased capacity to induce *in vitro* iTregs when compared with B6 and BALB/c mice. Lower expression of molecules involved in Treg suppressor mechanisms such as CD25, LAP-1, CD39 and PD-1 was observed both in NOD iTregs and Tregs from lymph nodes of NOD mice. Moreover, *in vitro* assays showed that Tregs from NOD mice exhibited reduced ability to suppress proliferation of CD4$^+$CD25- responder T cells when compared with B6 and BALB/c mice. Major differences were consistently observed between NOD and BALB/c mice, whereas no major differences were found for many of the analyzed parameters between the NOD and B6 mice, suggesting that highly and mildly autoimmune prone mouse strains may share some Tregs features. On the contrary, BALB/c Tregs were in major quantities, expressed higher levels of Foxp3 and exhibited more potent ability to inhibit effector T cell proliferation, data that could be related to its natural resistance to the induction of different experimental autoimmune conditions. Altogether our results demonstrate a generalized Treg cell dysfunction in NOD mice, a strain characterized by its high predisposition to develop spontaneous and induced autoimmune diseases.

1. Introduction

C57BL/6 (B6), BALB/c and NOD mice are among the most frequently used mouse strains in immunological research, exhibiting differential susceptibility to develop autoimmune responses. NOD mice develop type one diabetes (T1D), autoimmune thyroiditis (EAT), autoimmune prostatitis (EAP) and sialoadenitis spontaneously with age and are also prone to the induction of autoimmune encephalitis (EAE), EAT and EAP upon immunization [1–4]. B6 mice are susceptible to the induction of T1D, EAE, EAP, autoimmune uveitis (EAU) and myasthenia gravis (EAMG) [4–7]. On the contrary, BALB/c mice are more resistant to the induction of all these autoimmune conditions [4,7,8]. Thus, based on predisposition to develop autoimmunity, NOD, B6 and BALB/c mice can be classified as highly prone, mildly prone and resistant, respectively.

Lack of tolerance in autoimmune conditions has been postulated to at least partly result from ineffective T regulatory suppression of autoreactive T effector cells [9]. Indeed, increasing evidence has implicated CD4$^+$Foxp3$^+$ regulatory T cells (Tregs) in both the breakdown of self-tolerance and the restoration of immune homeostasis [10]. Treg depletion has been shown to accelerate autoimmune diabetes development, to induce an increment in prostatitis severity in NOD and B6

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mice, and, remarkably, to produce a switch of BALB/c mice from resistant to a susceptible phenotype [8,11,12]. On the contrary, transfer of Tregs or treatments that allow *in vivo* Treg expansion showed to reverse diabetes, EAU, EAE and EAMG in NOD or B6 mice [13–15].

Alterations in numbers and/or functionality of Tregs have been proposed to occur in the autoimmune-prone NOD mouse [10]. Quantitative deficiency of Tregs was reported in NOD mice, whereas other studies found normal levels of Tregs when compared with other mouse strains [16–19]. Alternatively, qualitative defects in suppressive function of Tregs was proposed as another mechanism for failed suppression in NOD mice [20], showing age-based decline in Treg cell functional potency [18]. However, a comparative analysis between NOD and B6 mice showed that Tregs were equally functional in both strains [16]. However, other reported data indicate that diabetes onset in NOD mice was only partially explained by defective Treg activity suggesting a progressive resistance of diabetogenic T effector cells to Treg inhibition [21,22]. In most of the studies mentioned above, Treg analysis were limited to the $CD4^+CD25^{hi}$ $Foxp3^+$ cell pool, without considering possible differences in Tregs subsets.

Compelling evidence indicates that Tregs consist of various subpopulations and have a more or less plastic phenotype depending on the microenvironment [23]. Tregs can be broadly classified into two groups on the basis of their developmental origin: thymus-derived Tregs (tTregs) that develop in the thymus from CD4 single positive thymocytes and in general display high affinity self-reactive TCRs, and peripherally induced Tregs (pTregs) that emerge in the periphery from conventional $CD4^+$ T lymphocytes in response to environmental antigens and tolerogenic stimuli [24]. The relative contribution of tTreg and pTreg in immune tolerance remains a major unveiled issue, partially due to the lack of specific markers to definitively distinguish them. However, some experimental data indicate that Helios or neuropilin-1 could be specific markers of tTregs [25,26]. Besides induced Tregs (pTregs) *in vivo*, $Foxp3^+$ Tregs can also be induced *in vitro* by TCR-mediated activation of naïve T cells in the presence of TGF-β and IL-2 (iTregs), although they show unstable Foxp3 expression and lacks a fraction of signature genes that distinguish *bonafide* tTregs [27]. Subpopulations of Tregs are also classified on their activation status. Indeed, most Tregs have naïve phenotype ($CD44^{low}CD62L^{hi}$), localize in secondary lymphoid organs, and have been termed central Treg (cTreg). In addition, activated/memory Tregs (mTregs) are characterized by the expression of $CD44^{hi}CD62L^{low}$, are extensively dividing cells expressing multiple activation markers, dominant in non-lymphoid organs and also present in secondary lymphoid tissues [23,28]. Once activated, Tregs exert suppressive functions though several mechanisms that include release of anti-inflammatory cytokines, cytolysis, expression of co-inhibitory molecules (such as CTLA-4, LAG-3, PD-1, PD-L1), antigen presenting cells modulation and metabolic disruption [29,30]. In addition, it has been demonstrated that Tregs have the ability to acquire specific features to the type of immune response they control, e.g. expressing the respective transcription factors and chemokine receptors to restrain Th1, Th2 and Th17 responses and to migrate to the appropriate location where they must fulfill their function [31,32].

In the present report, by comparing Tregs from most frequently used mouse strains in immunological research (B6, BALB/c and NOD), we provide evidence showing that the NOD mice exhibit a generalized decreased in Treg counts with enhanced proportions of $CD44^{hi}CD62L^{low}$ Tregs with respect to BALB/c mice. Naïve $CD4^+$CD25- T cells from NOD mice also showed decreased capacity to induce *in vitro* iTregs together with low expression of CD25, LAP-1, CD39 and PD-1, molecules involved in Treg functional suppressive mechanisms. Moreover, *in vitro* assays showed that Tregs from NOD mice exhibited reduced ability to suppress $CD4^+$CD25- responder T cells with respect to B6 and BALB/c mice. Altogether, our results demonstrate a generalized Treg cells dysfunction in NOD mice, a strain characterized by its high predisposition to develop spontaneous and induced autoimmune diseases.

2. Materials and Methods

2.1. Mice

Female, non-diabetic, 4-6-week old mice were used in all experiments. NOD, B6, and BALB/c mice were purchased from Jackson Laboratory and then bred and maintained under specific pathogen-free conditions in the animal facility of the Centro de Investigaciones en Bioquímica Clínica e Inmunología, Universidad Nacional de Cordoba, Argentina. Animals were maintained in a 16 h light-8 h dark cycle, at 20 ± 2 °C, with food and water ad libitum. All experiments were approved by and conducted in accordance with guidelines of the Committee for Animal Care and Use of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba (Res. HCD 240/216), in strict accordance with the recommendation of the Guide for the Care and Use of Laboratory Animals of the NIH (NIH publication 86-23). Animals were not subjected to any treatment. All efforts were made to minimize suffering and discomfort.

2.2. Flow cytometry

Single cell suspensions were prepared from the thymus, spleen, axillary and popliteal (pLN), pancreatic (pcLN), mesenteric lymph nodes (mLN) and Peyer Patches (PP) of individual mice and stained for surface markers. Dead cells were excluded using Live-dead fixable reagent (Invitrogen). The following antibodies from eBioscience (San Diego, CA, USA) or Biolegend (San Diego, CA, USA) conjugated with different fluorochromes were used: anti CD4, CD25 (PC61), GITR, CTLA-4, CD73, CD39, CD3, CD8, LAG-3, LAP-1, PD-1, PD-L1, CD44, CD62 L, Helios, CXCR3, CCR5 and CCR6. For intracellular Foxp3 and CTLA-4 staining, a Foxp3 Staining Kit (eBioscience) was used. Finally, cells were washed twice with 2% FBS-saline solution and stored at 4 °C in the dark. Cells were acquired in FACS-CANTO II or LSRFortessa flow cytometers (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (version 7.6.2). Proper compensation using Fluorescence Minus One (FMO) controls were used.

2.3. In vitro Treg generation

For iTreg generation, spleen mononuclear cell suspensions (MNCs) were obtained using Ficoll-Hypaque PREMIUM 1.084 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) centrifugation gradients. $CD4^+$ cells were enriched from splenic MNCs using EasySep™ Mouse $CD4^+$ T Cell Isolation Kit (StemCel Technologies, Vancouver, Canada). $CD4^+$ CD25-$CD62L^+$CD44- T cells were then sorted from a $CD4^+$ cell population using a FACSAria cell sorter (BD Biosciences, San Diego, CA, USA). Sorted cells were then incubated with plate-bound anti-CD3/CD28 (1 μg/ml and 0,25 μg/ml, respectively) (BD Biosciences), 100 U/ml rIL-2 and 5 ng/ml rhTGFβ (Peprotech, Rocky Hill, NJ, USA) for 5 days at 37 °C, 5% CO_2.

2.4. Cell purification and in vitro Treg suppression assay

Cells were obtained from pooled spleens subjected to mechanical tissue disruption. Mononuclear cell suspensions (MNCs) were prepared under sterile conditions in HBSS (Sigma-Aldrich, St. Louis, MO, USA) and separated on Ficoll-Hypaque PREMIUM 1.084 (GE Healthcare Bio-Sciences AB) centrifugation gradients. $CD4^+$ T cells were enriched from MNCs using EasySep™ Mouse $CD4^+$ T Cell Isolation Kit (StemCell Technologies). Finally, $CD4^+CD25^{hi}$ (Treg) and $CD4^+$CD25- cells were sorted from a $CD4^+$cell population using a FACSAria cell sorter (BD Biosciences), and on the basis of CD4 and CD25 expression. $FoxP3^+CD4^+CD25^{hi}$ T cell population purity was higher than 97%, evaluated by intracellular staining with the FJK-16 s mAb (eBioscience).

The suppressive ability of $CD4+CD25^{hi}$ T cells from all mouse

strains was tested by culturing syngeneic CFSE-labeled $CD4^+CD25$- T responder cells with $CD4^+CD25^{hi}$ T cells. In brief, different ratios of FACS-sorted $CD4^+CD25^{hi}$ T cells were cultured with CFSE-labeled $CD4^+CD25$- T cells in 96-well round bottom plates-bound anti-CD3/CD28 (2 μg/ml and 1 μg/ml, respectively), (BD Biosciences) in triplicates for 3 days. Proliferation was assessed by FACS analysis as the total percentage of CFSE labeled $CD4^+CD25$- T cells undergoing at least one round of division.

2.5. Statistical analysis

Statistical analysis was performed using one or two-way ANOVA with Bonferroni post hoc test analysis. Data are shown as mean ± SEM. Statistical tests were performed using the GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, CA, USA). *P* values * < 0.05, ** < 0.01, *** < 0.001 and **** < 0.0001 were considered significant in all analyses.

3. Results

3.1. NOD mice exhibit decreased Treg cell counts and Foxp3 expression in lymphoid tissues

As introduced above, contradictory findings regarding quantitative and qualitative deficiencies of Tregs in autoimmune prone NOD mice have been reported, being most of them obtained by comparing NOD and B6 mice [16–19]. In the present work, we analyzed $CD4^+CD25^+Foxp3^+$ Tregs frequencies and absolute numbers in NOD, B6 and BALB/c mice. We analyzed Tregs from different lymphoid tissues in young (4-6-week old) mice from our animal facility colony to ensure they were neither pre diabetic nor had T-cell infiltration in the pancreas or other tissues. Significantly reduced total counts of $CD4^+Foxp3^+$ T cells were observed in NOD mice with respect to BALB/c mice in spleen, pLN, pcLN, mLN and PP. Frequencies of $CD4^+Foxp3^+$ T cells were also reduced although in PP percentages showed no differences between strains (Fig. 1A-C). In addition, Tregs frequencies in pLN and pcLN of NOD mice were also significantly lower than those observed in B6 mice (Fig. 1A-B). Moreover, decreased levels of Foxp3 expression were also observed in NOD Tregs. Indeed, when the relative expression of Foxp3 on Tregs from different lymphoid tissues was analyzed, Tregs from the spleen, pLN, mLN and PP of NOD mice showed to express significantly lower protein levels than Tregs from BALB/c mice. No differences were detected in Tregs from the thymus and pcLN among NOD mice and the other mouse strains under study (Fig. 1D-E). Altogether, these results indicate that NOD mice have lower numbers of Tregs with also decreased Foxp3 expression in different lymphoid tissues, being these differences more pronounced with respect to BALB/c mice.

3.2. NOD, B6, and BALB/c mice have different proportions of thymic/peripheral, central/memory Treg cell subsets

It has been proposed that Helios is a marker of thymic-derived Tregs (tTregs), while Helios-Tregs are induced from Foxp3- T conventional cells in the periphery (pTregs) [25]. We then analyzed if mouse strains that are highly-prone, mildly-prone or resistant to develop autoimmunity exhibit different proportions of $Helios^+$ and Helios– Tregs in steady state conditions. Whereas proportions of $Helios^+$ and Helios- Tregs showed no differences in the spleen and mLN between mouse strains under study, lower frequencies of $Helios^+$ Tregs were observed in pLN of NOD and B6 mice with respect to BALB/c mice (Fig. 2A-B).

The analysis of $CD44^{low}CD62L^{hi}$ Tregs (cTregs) and $CD44^{hi}CD62L^{low}$ Tregs (mTregs) showed enhanced proportions of $CD44^{hi}CD62L^{low}$ Tregs in the spleen and pLN from NOD mice with respect to B6 and BALB/c mice (Fig. 2 C-D). In accordance, significantly reduced frequencies of $CD44^{low}CD62L^{high}$ Tregs were found in the spleen from NOD mice with respect to B6 and BALB/c mice (Fig. 2 C-D). No differences were observed in cTregs or mTregs frequencies in mLN among the mouse strains under study (Fig. 2C-D). Altogether, our data indicate that NOD mice have lower Tregs counts and also lower proportions of tTregs and cTregs when compared with BALB/c mice.

3.3. Phenotypic characteristics of Tregs in NOD, B6 and BALB/c mice in steady state conditions

Since Tregs exert suppressive functions through mechanisms that include functional molecules like CD25, LAP-1, CD73, CD39, PD-1, CTLA-4 and others [33], we then evaluated their expression levels in splenic and pLN Tregs from all mouse strains under study. On the one hand, and as shown in Fig. 3, splenic Tregs from the three mouse strains showed no major differences in the expression levels of the analyzed molecules. We observed lower levels of expression of CD25 in NOD Tregs than in BALB/c and B6 Tregs (Fig. 3A and C). In addition, Tregs from NOD and BALB/c mice showed significantly lower levels of CD73 with respect to Tregs from B6 mice. Conversely, the highest expression of PD-1 was detected in Tregs from NOD mice (Fig. 3A and B). On the other hand, the analysis of these molecules in Tregs from pLN showed more marked differences among the mouse strains studied (Fig. 3B and C). Again, CD25 expression was significantly lower in Tregs from NOD mice with respect to B6 and BALB/c mice (Fig. 3B and C). Also, levels of expression of LAP-1, CD39 and PD-1 were lower in Tregs from NOD mice than those found in Tregs from B6 and BALB/c mice. Moreover, while CD73 showed the highest expression levels in Tregs from B6 mice, no differences were observed for CTLA-4, GITR and PD-L1 in spleen and pLN Tregs from the three mouse strains under study (Fig. 3A-C). These results indicate that no major differences in the expression of molecules associated with suppressive activity of Tregs are observed in steady state conditions among the mouse strains under study. However, and noteworthy, Tregs from the NOD mice, mainly those present in lymph nodes, showed reduced levels of expression of CD25 and other inhibitory molecules.

3.4. Differential expression of chemokine receptors associated to suppression on specific effector T cell subsets

It has been described that different subsets of Tregs acquire the expression of particular chemokine receptors to restrain Th1 or Th17 responses and to migrate to the appropriate location where they must accomplish their function [34]. It has been shown that CCR6 is preferentially expressed on Tregs that control Th17 cells, whereas CXCR3 and CCR5 on Tregs that restrain Th1 cells [34]. We then investigated CCR6, CXCR3 and CCR5 expression on Tregs from all mouse strains under study (Fig. 4). Similar proportions of $CXCR3^+$ and $CCR6^+$ Tregs were detected in the spleen from all mouse strains, while lower $CCR5^+$ Tregs were detected in the spleen from NOD mice when compared with B6 and BALB/c (Fig. 4A-B). The analysis of chemokine receptor expression on Tregs form lymph nodes showed lower proportions of $CXCR3^+$ and CCR5+ Tregs in NOD mice when compared with BALB/c mice (Fig. 4C-D). Altogether, these data indicate that NOD mice, which are more susceptible to develop autoimmune diseases mediated by pathogenic Th1 cells, exhibit lower proportions of $CXCR3^+$ and $CCR5^+$ Tregs.

3.5. NOD mice exhibit reduced capacity to induce $Foxp3^+$ cells in vitro

Sorted $CD4^+CD25$- naïve conventional T cells from NOD, B6 and BALB/c mice were stimulated with plate-bound anti-CD3/CD28 in the presence of IL-2 and TGF-β for 5 days, which are standard conditions for Foxp3 induction [35]. Although a considerable induction of $Foxp3^+$ cells was observed in all mouse strains, NOD mice showed the lowest proportions of induced $Foxp3^+$ cells (Fig. 5A). Conversely, BALB/c mice cells showed the higher ability to induce Foxp3 expression when

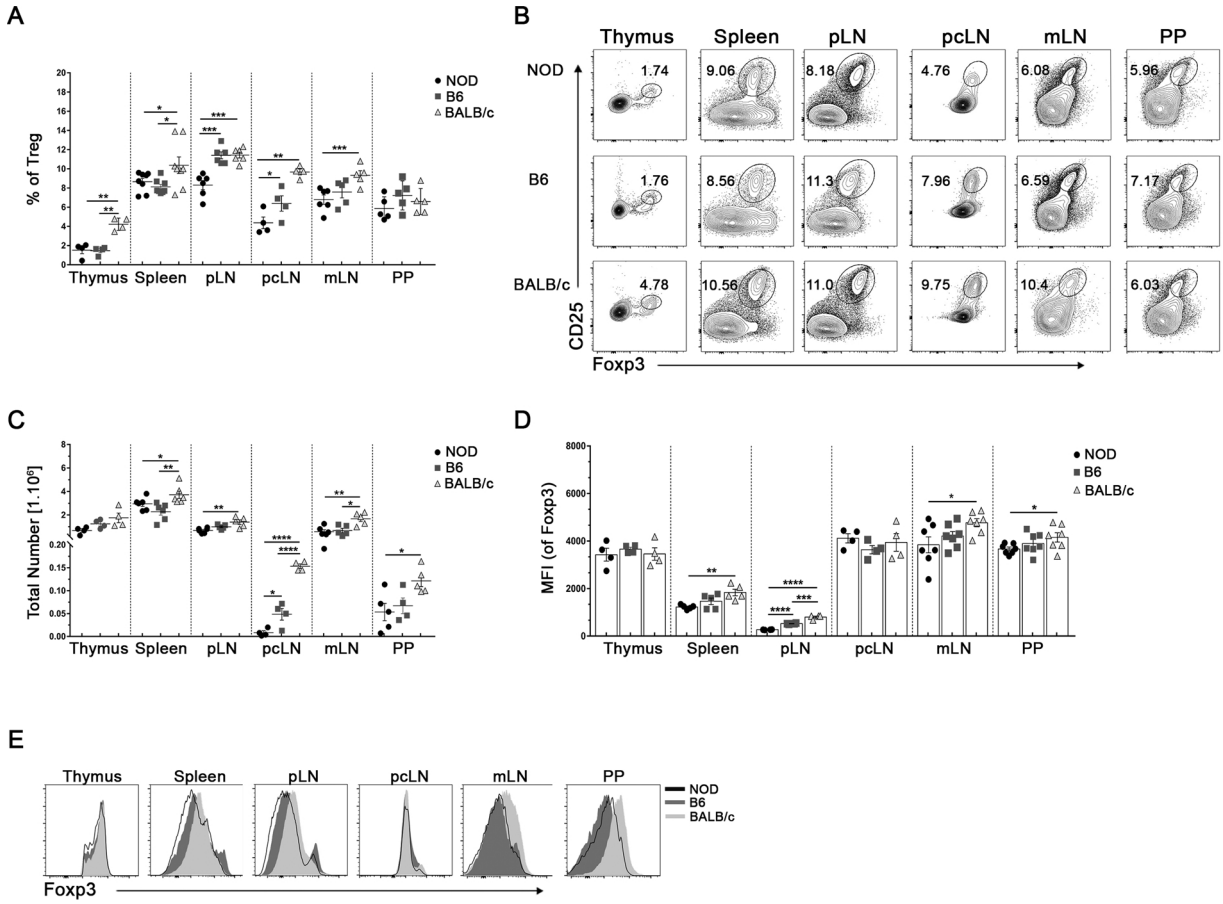

Fig. 1. NOD mice exhibit decreased Treg cell counts and Foxp3 expression in lymphoid tissues.
(A) Dot graphs and (B) representative contour plots showing Foxp3$^+$ CD25$^+$ cell frequencies in the CD4$^+$ cell population of the thymus, spleen, pooled inguinal and popliteal lymph nodes (pLN), pancreatic lymph nodes (pcLN), mesenteric lymph nodes (mLN) and Peyer patches (PP) from 4-6-week old NOD, B6 and BALB/c mice. (C) Absolute numbers of Treg cells in the thymus, spleen, pLN, pcLN, mLN and PP from NOD, B6 and BALB/c mice. (D) Bar graph showing Mean Fluorescence Intensity (MFI) values for Foxp3 expression in Tregs from the thymus, spleen, pLN, pcLN, mLN and PP from NOD, B6 and BALB/c mice. (E) Representative histograms of Foxp3 expression in Tregs from all tissues analyzed. Gates were performed on viable cells using Live-dead fixable (Invitrogen) dye and Fluorescence Minus One (FMO) controls. Data are shown as mean ± SEM, n = 4-8 mice per group, and are representative of three independent experiments with essentially the same results. The *p* values were obtained using one-way ANOVA followed by Bonferroni post-hoc analysis as appropriate. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

compared with NOD mice (Fig. 5A). In addition, Foxp3 and CD25 expression levels were more elevated in iTregs from BALB/c mice when compared with B6 and NOD mice (Fig. 5B). When we analyzed the expression levels of different molecules involved in suppressive mechanisms, lower levels of expression of CTLA-4, LAP-1, CD39, PD-L1 and LAG-3 were detected in iTregs from NOD mice when compared with iTregs from BALB/c mice. The only molecule that showed higher expression in NOD Tregs was GITR. Interestingly, no differences in CTLA-4, CD39, PD-L1 and LAG-3 expression levels were observed between NOD and B6 mice (Fig. 5B). However, iTregs of B6 mice showed higher expression levels of CD73 (Fig. 5B), similar to what was observed in splenic and pLN Tregs in steady state conditions (Fig. 3). These data indicate that NOD mice iTregs have a reduced ability to be induced *in vitro*. Moreover, these iTregs express less levels of Foxp3, CD25and other molecules that exert immunosuppression.

3.6. Tregs from NOD mice have reduced ability to suppress effector T cell proliferation

It has been established that immunosuppressive ability of Tregs could be evaluated by *in vitro* inhibition of naïve CD4$^+$CD25- T cell proliferation [29]. We then conducted experiments in which spleen derived Tregs from all mouse strains under study were co-cultured with CFSE-labeled CD4$^+$CD25- effector T cells stimulated with plate-bound anti-CD3/CD28 for 3 days. As shown in Fig. 6A-B, the most potent ability to suppress the proliferation of effector T cells was shown by Tregs from BALB/c mice, for every Tconv/Treg cell ratio analyzed. In addition, Tregs from B6 mice showed the ability to suppress effector T cell proliferation at 4:1, 2:1 and 1:1 Tconv/Treg cell ratios, whereas Tregs from NOD mice were able to suppress T cell proliferation only when cultured at 2:1 and 1:1 Tconv/Treg cell ratios (Fig. 6A-B). These results indicate that Tregs from BALB/c and NOD mice have the highest and lowest immunosuppressive ability, respectively.

4. Discussion

Effective regulatory function by Tregs is undoubtedly a key issue for immune homeostasis [36]. In patients with IPEX and *scurfy* mice, the complete absence of Tregs is known to cause a severe and lethal multi-organ disease [37]. Therefore, it is likely that lower numbers and/or less than optimal function could have a significant impact on the function of the immune system thereby contributing to the development of autoimmune diseases [38]. Factors that control Tregs numbers and functions have not been completely elucidated, but it is well known that genetic components have a significant influence [39].

In the present report, by conducting a comparative study between young NOD, B6 and BALB/c mice, we provide data showing that NOD mice have markedly reduced Treg numbers, Foxp3 and CD25

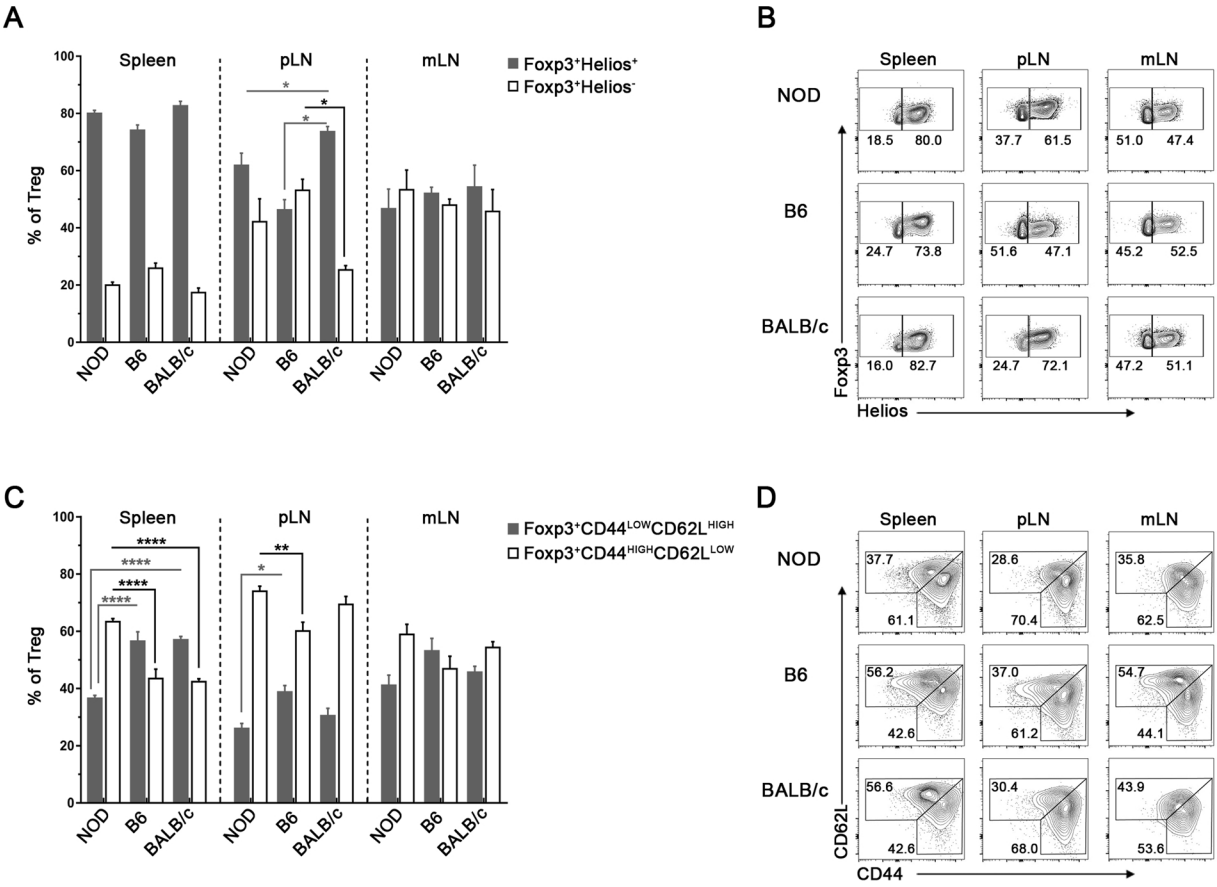

Fig. 2. Proportions of Helios$^+$, CD44-CD62L^{hi} and CD44^{hi}CD62L- Treg cell subsets in NOD, C57BL/6 and BALB/c mice.
(A) Bar graphs and (B) representative contour plots showing the frequencies of Foxp3$^+$ Helios$^+$ and Foxp3$^+$ Helios- Tregs in the CD4$^+$ Foxp3$^+$cell population of the spleen, pLN and mLN from 4-6-week old NOD, B6 and BALB/c mice. (C) Bar graphs and (D) representative contour plots showing the frequencies of CD62L$^+$ and CD44$^+$ cells in the CD4$^+$ Foxp3$^+$ cell population of the spleen, pLN and mLN from 4-6-week old NOD, B6 and BALB/c mice. Gates were performed on viable cells using Live-dead fixable (Invitrogen) dye and Fluorescence Minus One (FMO) controls. Data are shown as mean ± SEM, n = 4-5 mice per group, and are representative of three independent experiments with essentially the same results. The *p* values were obtained using one-way ANOVA followed by Bonferroni post-hoc analysis as appropriate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

expression in different lymphoid tissues. Most differences were consistently observed between NOD and BALB/c mice, while for many of the analyzed parameters no major differences were found between NOD and B6 mice, suggesting that highly and mildly autoimmune prone mouse strains may share some Tregs features. Our results add to the current knowledge of Tregs and could explain the controversial available evidence about NOD Treg quantities and quality, since most of the reported data was obtained from studies that just compared NOD and B6 mice [16–18,40]. Interestingly, our data show that BALB/c mice have higher counts of Tregs, express higher levels of Foxp3 and show a more potent ability to restrain effector T cell proliferation; data that could be related, at least in part, to the natural resistance of this mouse strain to develop autoimmunity [4,7,8] and to their high susceptibility to develop tumors [41–43].

Several subsets of Tregs with distinct origin, activation state, phenotypes and effector functions have been identified [29,44]. We investigate if besides having reduced numbers, NOD mice showed differences in Tregs of different origins that may play non redundant roles in controlling autoimmunity [45]. Some researchers have proposed that tTregs may ensure tolerance to self-antigens due to their differentiation in thymus guided by TCRs that recognize self-antigens [46], while pTregs may represent TCRs specificities to antigens derived from commensal microbiota, diet or pathogens [47]. It could be speculated that high susceptibility to autoimmune responses could be associated to reduced tTreg counts. Indeed, our data showed low proportions of Helios$^+$ Treg in pLN, but not in the spleen or mLN, from NOD mice. However, the use of Helios to discriminate between tTregs and pTregs has been questioned and no consensus has been reached until now [48]. Interestingly, NOD mice deficient for the Foxp3 enhancer CNS1 (known to be involved in pTreg induction), showed decreased pTreg frequencies but also increased risk of T1D, suggesting that pTregs accomplish an important non-redundant function in preventing autoimmune diabetes [49]. In addition, Ferreira et al. reported that tTregs of NOD mice have a defective and reduced TCR diversity despite having normal cellularity in the thymus [50]. Thus, a deeper analysis of quantities and specificities of the pTregs and tTregs subsets is currently needed to elucidate the relative contribution of each subset to immune tolerance.

Regulatory T cells are also sub grouped as either naïve/central or effector/memory by their developmental stage and phenotype [23]. It has been postulated that naïve/central Tregs localize in secondary lymphoid organs and effector/memory Tregs have the ability to home into inflamed sites being proposed as sentinels of tolerance [28]. In our experiments enhanced proportions of CD44^{hi}CD62L^{low} Tregs were detected in the spleen and pLN of NOD mice with respect to B6 and BALB/c mice. Our results are in agreement with those reported by others that found decreased frequencies of CD44^{low}CD62L^{hi} Tregs [51,53]. Indeed, using adoptive transfer of CD62L^{low} or CD62L^{hi} Tregs, Szanya et al. showed that CD62L^{hi} Tregs was the subpopulation with the highest capability to delay the onset of diabetes. The latter suggests that the reduced CD62L^{low} Tregs counts observed in our setting could be favoring autoimmunity development in NOD mice [52].

Regulatory T cells can also adopt distinct subphenotypes that differ

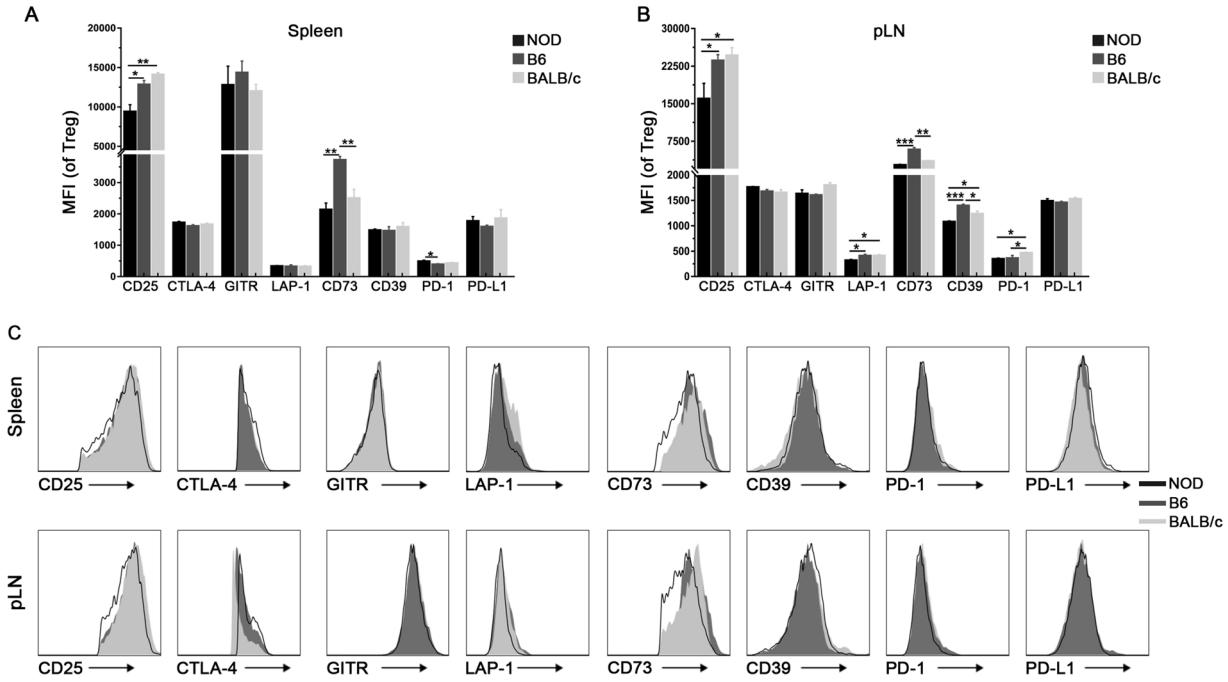

Fig. 3. Phenotypic characteristics of Tregs in NOD, B6 and BALB/c mice in steady state conditions.
(A-B) Bar graphs showing Mean Fluorescence Intensity (MFI) values for CD25, CTLA-4, GIRT, LAP-1, CD73, CD39, PD-1 and PD-L1 in $CD4^+Foxp3^+$ splenic Tregs (A) and $CD4^+Foxp3^+$ cells of a pool of inguinal and popliteal lymph nodes (pLN) (B) from 4-6-week old NOD, B6 and BALB/c mice. (C) Representative histograms for the above molecules in $CD4^+Foxp3^+$ Tregs from Spleen and pLN. Gates were performed on viable cells using Live-dead fixable (Invitrogen) dye and Fluorescence Minus One (FMO) controls. Data are shown as mean ± SEM, n = 4-5 mice per group, and are representative of three independent experiments with essentially the same results. The *p* values were obtained using one-way ANOVA followed by Bonferroni post-hoc analysis as appropriate. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

by their preferential expression of chemokine receptors, effector molecules, and cofactors that collaborate with Foxp3 to drive their functional capabilities [53]. These sub-phenotypes often are driven by transcription factors central to the differentiation and effector functions of the cells they regulate. For instance, Tbet and CXCR3 are induced in Tregs that regulate type 1 inflammation [54]. Interestingly, NOD mice are susceptible to developing autoimmune diseases that are mostly induced by pathogenic Th1 cells. In agreement, our data showed that NOD have lower proportions of Tregs expressing CXCR3 and CCR5 than BALB/c mice.

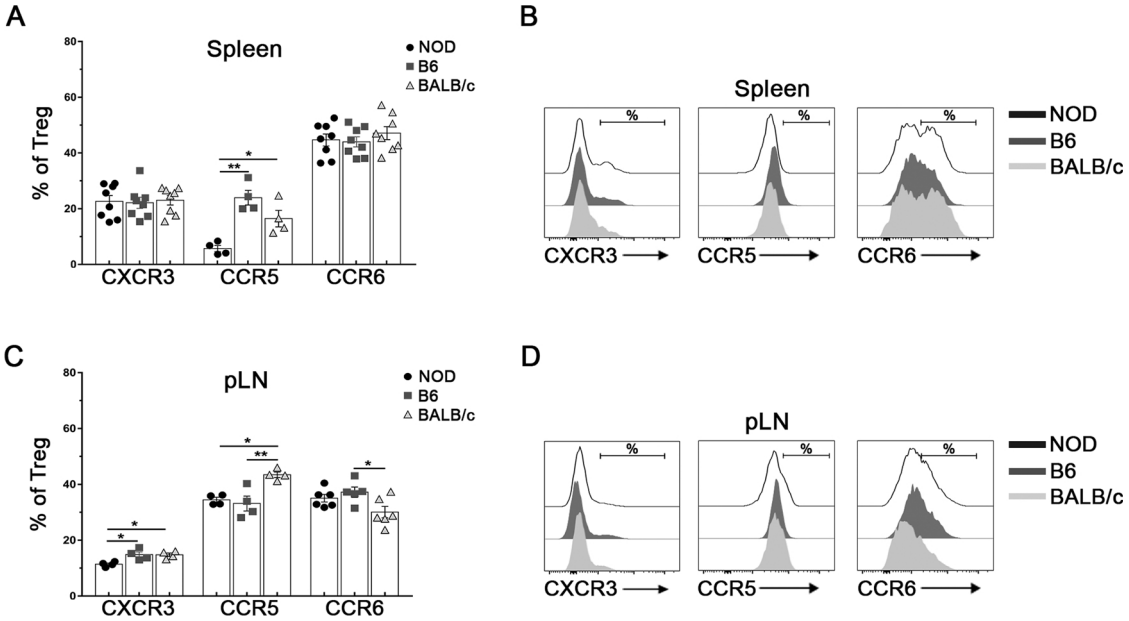

Fig. 4. Proportions of CCR6+, CXCR3+ and $CCR5^+$ Treg subsets in NOD, B6 and BALB/c mice.
(A) Bar graphs and (B) Representative histograms for $CXCR3^+$, $CCR5^+$ and $CCR6^+$ in in the $CD4^+$ $Foxp3^+$ cell population of the spleen from 4-6-week old NOD, B6 and BALB/c mice. (C) Bar graphs and (D) Representative histograms for $CXCR3^+$, $CCR5^+$ and $CCR6^+$ in the $CD4^+$ $Foxp3^+$ cell population of a pool of inguinal and popliteal lymph nodes (pLN) from NOD, B6 and BALB/c mice. Gates were performed on viable cells using Live-dead fixable (Invitrogen) dye and Fluorescence Minus One (FMO) controls. Data are shown as mean ± SEM, n = 4-5 mice per group, and are representative of three independent experiments with essentially the same results. The *p* values were obtained using one-way ANOVA followed by Bonferroni post-hoc analysis as appropriate. $*p < 0.05$ and $**p < 0.01$.

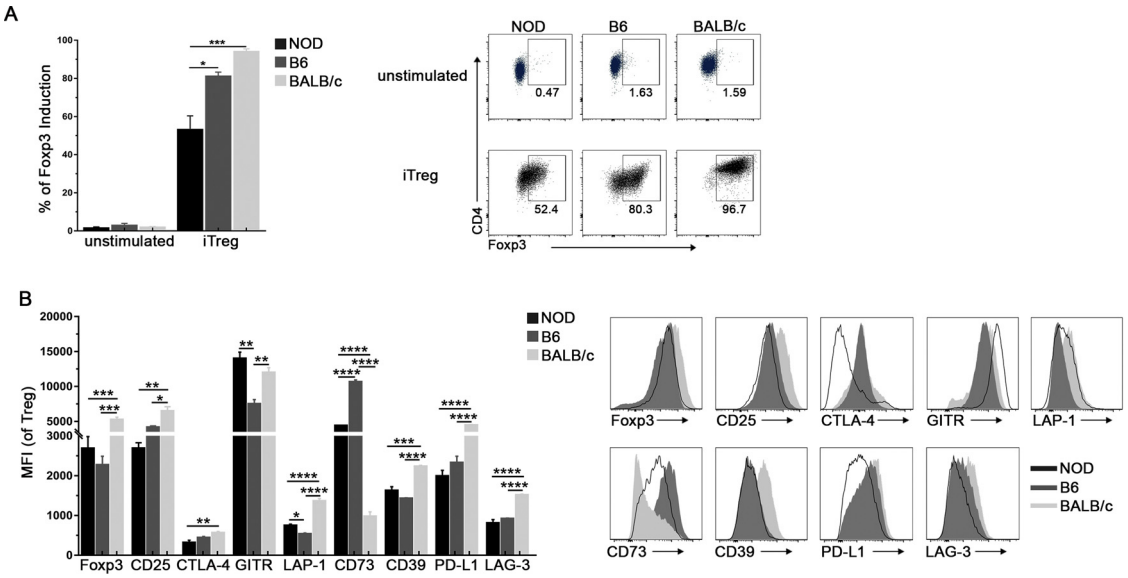

Fig. 5. NOD mice show a reduced capacity for iTreg generation.
(A) Bar graphs (left) and representative dot plots (right) respectively showing the frequencies of Foxp3$^+$CD4$^+$ cells and Foxp3 expression, after culturing CD4$^+$CD25- CD62L$^+$CD44- sorted cells from NOD, B6 and BALB/c with αCD3/CD28 + rIL-2/rhTGF-β. (B) Bar graphs and representative histograms showing Mean Fluorescence Intensity (MFI) values and Foxp3, CD25 CTLA-4, GITR, LAP-1, CD73, CD39, PD-L1 and LAG-3 expression in splenic iTregs from NOD, B6 and BALB/c mice. Gates were performed on viable cells using Live-dead fixable (Invitrogen) dye and Fluorescence Minus One (FMO) controls. To purify CD4$^+$CD25- cells, 12 mice of each mouse strain were used. Data correspond to mean ± SEM, of three independent experiments. The *p* values were obtained using one-way ANOVA followed by Bonferroni post-hoc analysis as appropriate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. and ****$p < 0.0001$.

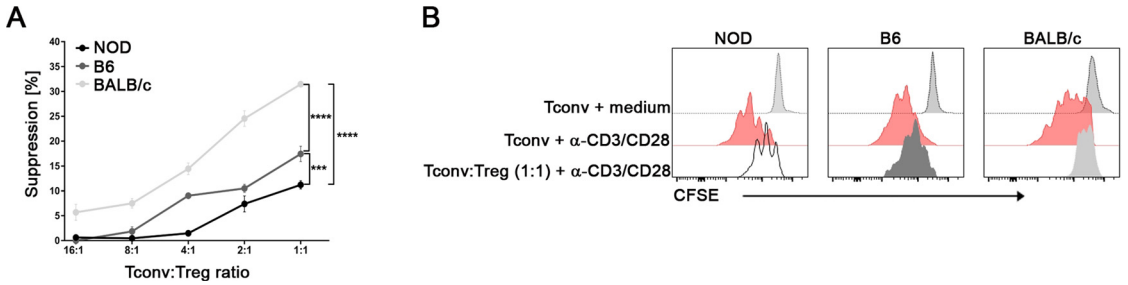

Fig. 6. Treg from NOD mice have reduced capability to suppress effector T cell proliferation.
(A) Percentage of suppression of CD4$^+$CD25- (Tconv) cell proliferation at different Tconv:Treg cell ratios from NOD, B6 and BALB/c mice. (B) Representative histograms for CFSE fluorescence in gated CD4$^+$ T cells from NOD, B6 and BALB/c mice after αCD3/CD28 stimulation alone or in the presence of equal amounts of CD4$^+$CD25- (Tconv) and CD4$^+$CD25^{hi} (Treg) cells (Tconv:Treg 1/1). To purify Tconv and Treg cells, 12 mice of each mouse strain were used. Data correspond to mean ± SEM, of at least three independent experiments. The *p* values were obtained using one-way ANOVA followed by Bonferroni post-hoc analysis as appropriate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. and ****$p < 0.0001$.

As mentioned before, Foxp3 expression can be turned on in conventional T cells as a consequence of antigen exposure in the periphery, producing the so-called pTregs that participate in the control of immunity, especially at the mucosal surfaces [55]. Moreover, naive CD4$^+$ Tconv cells activated *in vitro* in the presence of IL-2 and TGFβ induce Foxp3 (iTreg) [35]. Although iTregs do not recapitulate the functional or phenotypic characteristics of in vivo generated pTregs, it could be speculated that the ability to induce iTregs could be a measure of the capability to generate pTregs *in vivo*. Herein, we provide data showing that naïve T cells from NOD mice exhibited the lowest ability to induce Foxp3$^+$ expression whereas BALB/c mice showed the highest ability to induce Foxp3 expression under standard conditions for Foxp3 induction. Moreover, iTregs from NOD mice express less Foxp3 and other inhibitory molecules that exert immunosuppression indicating that NOD iTregs are deficient in quantity and quality.

Regulatory T cells exert their function by secreting anti-inflammatory cytokines, consuming and thus depleting available IL-2 to restrain effector T cells by metabolic disruption, inducing dendritic cells to produce indoleamine 2,3- dioxygenase to suppress effector T cells, and other strategies [56]. Therefore, it is likely that lower expression of different molecules involved in Tregs regulatory mechanisms could have significant impacts on Tregs function. Indeed, in steady state conditions, low CD25, LAP-1, CD39 and PD-1 expression was observed in Tregs from lymph nodes of NOD mice. Moreover, Tregs from BALB/c and NOD mice respectively showed the highest and lowest capability to suppress T cell proliferation. IL-2 is an essential factor for Tregs maintenance and in its absence a profound deficiency of Tregs is observed [57]. One of the genetic regions associated with NOD autoimmune susceptibility is that one that includes the IL-2 encoding gene, linked to reduced IL-2 expression and probably related to alterations in Treg cell pools [58,59]. It has been demonstrated that NOD mice and also patients with type 1 diabetes exhibit decreased pSTAT5 response due to alterations in the level of expression of molecules involved in IL-2R signaling [60,61]. Taking into account the IL-2 dependence for Tregs maintenance, it is possible that low levels of IL-2 and also defects in IL-2R signaling could be both contributing to the generalized Treg cell dysfunction observed in NOD mice.

The essential role of Tregs in the control of autoimmune responses endows them with tremendous therapeutic potential and has led to intensive research focused on increasing their quantities and/or

functionality. Administration of IL-2 at low doses has been shown to suppress immune pathologies by preferentially expanding Tregs [62]. However, this therapy has been limited by off-target complications due to pathogenic cell expansion. Recent efforts have been focused on developing more selective IL-2 treatments using certain complexes of IL-2 plus anti-IL-2 antibodies that induce conformational changes resulting in selective targeting of Tregs and allowing a preferential STAT5 phosphorylation of Tregs *in vitro* and selective expansion of Tregs *in vivo*. Interestingly, the mentioned approach was able to induced remission of type 1 diabetes in the NOD mice [62].

Autoimmunity in NOD mice could be attributed to several different events occurring in the thymus and in the periphery. In addition to our data and those of other authors reporting a generalized Treg cell dysfunction, it has also been shown that NOD mice have defects in negative selection, perturbed αβ/γδ lineage decision leading to a shift in selection niches, reduced relative diversity of thymic Tregs, peripheral hyper-responsiveness of effector $CD4^+$ T cells, multiple binding registers of insulin peptides resulting in poor negative selection in the thymus and peripheral post-translational modification of self-peptides/neo-antigens [21,22,50,63–65]. The susceptibility to develop multiple autoimmune diseases in the NOD background is a unique opportunity to examine susceptibility features and genes that confer a general propensity for autoimmunity versus susceptibility genes that control individual autoimmune diseases.

5. Conclusions

From the comparative analysis of numbers, subsets, phenotype and function of Tregs from NOD, B6 and BALB/c mice, our results indicate that NOD mice, which are highly susceptible to develop autoimmunity, exhibit a generalized decrease in Treg cell numbers, lower expression of Foxp3 and other molecules involved in their function, decreased capacity to induce iTregs and also Tregs with reduced ability to suppress effector T cells. B6 mice, a strain in which it is possible to induce several autoimmune diseases upon immunization with autoantigens and adjuvants, showed an intermediate pattern with major Tregs numbers only in some tissue locations and similar Foxp3 expression indicating that Tregs from a mildly susceptible autoimmune strain shares some features with NOD Tregs. Finally, BALB/c mice, which are resistant to develop autoimmunity, showed higher numbers of Tregs, higher expression of Foxp3 and other molecules associated with their function, enhanced capability to induce iTregs, and Tregs that strongly suppressed proliferation of effector T cells. Our results indicate a generalized Treg cell dysfunction in NOD mice that associates with their natural susceptibility to develop spontaneous and induced autoimmune conditions. Altogether, our results suggest that lower Treg cell numbers and/or less than optimal function could have significant impacts on immune homeostasis, thereby contributing to increase the susceptibility to develop autoimmune diseases.

Conflict of interest

The authors declare no financial or commercial conflicts of interest to disclose.

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