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B Cells Drive Autoimmunity in Mice with CD28-Deficient Tregs

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Abstract

Follicular regulatory T (T_{FR}) cells are a newly defined T regulatory (Treg) cell subset that suppresses follicular helper T (T_{FH}) cell-mediated B cell responses in the germinal center reaction. The precise costimulatory signal requirements for proper T_{FR} cell differentiation and function are still not known. Using conditional knockout strategies of CD28, we previously demonstrated that loss of CD28 signaling in Treg cells caused autoimmunity in mice (termed CD28- Treg mice), characterized by lymphoadenopathy, accumulation of activated T cells, and cell-mediated inflammation of the skin and lung. Here, we show that CD28 signaling is required for T_{FR} cell differentiation. Treg cell-specific deletion of CD28 caused a reduction in T_{FR} cell numbers and function that resulted in increased germinal center B cells and antibody production. Moreover, residual CD28-deficient T_{FR} cells showed a diminished suppressive capacity as assessed by their ability to inhibit antibody responses in vitro. Surprisingly, genetic deletion of B cells in CD28- Treg mice prevented the development of lymphoadenopathy and $CD4^+$ T cell activation, and autoimmunity that mainly targeted skin and lung tissues. Thus, autoimmunity occurring in mice with CD28-deficient Tregs appears to be driven primarily by loss of T_{FR} cell differentiation and function with resulting B cell driven inflammation.

Introduction

$Foxp3^+$ Treg cells (Tregs) are essential for immune homeostasis and prevention of autoimmunity (1). Constitutive loss of functional Tregs in humans or mice leads to severe autoimmunity shortly after birth (2–4). Moreover, induced loss of $Foxp3^+$ Tregs in adult animals leads to rapid autoimmune inflammation in adult animals (5), demonstrating the importance of these cells for both developing and maintaining immune self-tolerance.

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Thymic Treg development requires the integration of many signals from cell surface receptors, including the IL-2R and CD28 (6), which are important for effector T cell maturation as well. Loss of IL-2 or key component(s) of the IL-2R impairs Treg formation and leads to subsequent autoimmunity. Similarly, $CD28^{-/-}$ mice exhibit a loss of Tregs(7), although the impairment of effector T cells occurring as a result of loss of the CD28 costimulatory pathway prevents the emergence of rampant autoimmunity (8).

As CD28 is required for normal Treg development, it was difficult if not impossible to examine its role in Treg maintenance and function using mice with constitutive deletion of CD28. Thus, we created animals with targeted loss of CD28 in Tregs (termed CD28- Treg mice - (9)). Using these animals we showed a T cell intrinsic role for CD28 in mature Tregs, as CD28- Treg mice exhibited a number of autoimmune features, including skin and lung inflammation.

Tregs have important roles in suppressing both cellular and humoral responses (10), and in particular, Treg cells may directly suppress B cells and autoantibody generation to prevent autoimmune diseases (11, 12). Treg cells are able to kill B cells or migrate to germinal centers (GCs) where they suppress T helper cell-dependent B cell responses (13, 14). The likely mechanism for this mode of action is via a newly recognized Treg subset, named follicular Treg (T_{FR}) cells which express CXCR5, Bcl-6, ICOS and PD-1, share developmental cues with T follicular helper (T_{FH}) cells, and restrain GC reactions (10, 15–17).

The role of CD28 in Treg maintenance and function, together with the importance of T_{FR} cells in controlling GC reactions and antibody production led us to determine how loss of CD28 in $Foxp3^+$ cells altered T_{FR} cell differentiation and function. Here we show that CD28 is required for full T_{FR} cell differentiation as well as their optimal suppressive capacity. CD28- Treg mice display elevated numbers of T_{FH} cells, reduced numbers of T_{FR} cells, and enhanced responses to antigen immunization. Moreover, in vitro assays that control for T_{FR} cell numbers demonstrated reduced T_{FR} function on a per cell basis. Surprisingly, we found that while genetic ablation of B cells had only a minimal effect on the appearance of activated $CD8^+$ T cells in CD28- Treg mice, it prevented both the accumulation of activated $CD4^+$ T cells and the occurrence of autoimmunity. These data reveal an unexpected contribution of B cells towards autoimmunity seen when CD28 is targeted on Tregs, and suggest a critical role of the T_{FR}-T_{FH}-B cell axis in this process.

Materials and methods

Mice

Mice with conditional targeting of CD28 in $Foxp3^+$ cells ($CD28^{fl/fl}$ $Foxp3^{YFP\text{-}Cre}$, termed CD28- Treg mice, and female $CD28^{fl/fl}$ $Foxp3^{YFP\text{-}Cre/+}$) were generated and bred in our facility (9). B cell-deficient μMT B6 mice (19) were purchased from The Jackson Laboratory and bred to CD28- Treg mice. All colonies were maintained under specific pathogen-free conditions. All experiments described in this manuscript were approved by the Institutional Animal Care and Use Committee at the Massachusetts General Hospital.

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Media, reagents, antibodies, and flow cytometry

Cell cultures were performed using RPMI 1640 (Mediatech Inc.) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 50 mM 2-mercaptoethanol (Sigma-Aldrich). Fluorescent anti-CD3, anti-CD4, anti-CD8α, anti-CD19, anti-CD25, anti-CD38, anti-CD44, anti-CD62L, anti-CD69, anti-CD80, anti-IgM, anti-IgG, anti-IL-4 and anti-IL-17 antibodies were purchased from Biolegend. Anti-CTLA-4, anti-PD-1, anti-Fas, anti-ICOS, and anti-CXCR5 were purchased from BD Biosciences-Pharmingen. Anti-MHCII, anti-GL7, anti-IFNγ, anti-IL21 and an anti-Foxp3 staining kit were purchased from eBioscience. BD Cytofix/Perm buffer was used for intracellular staining. For cytokine detection, cells were stimulated by Leukocyte Activation Cocktail (BD Biosciences) for 4 to 6 hours and then fixed in eBioscience Fix/Perm buffer for intracellular cytokine staining. Cells were run on a BD LSR II flow cytometer or a Beckman Coulter Navios flow cytometer and analyzed by Flowjo (Flowjo LLC).

Histology

Freshly isolated tissues were fixed overnight at 4˚C in 10% neutral buffered formalin. Tissues were then rinsed in 70% ethanol and were sectioned by the DF/HCC Pathology Core for H & E staining. Immunohistochemical analysis was performed at the Dana-Farber Harvard Cancer Center Rodent Histopathology Core described as previously (9). Tissue sections were incubated with antibodies against mouse B220 and IgM (BD Bioscience), followed by biotinylated secondary antibodies (Vector).

NP-OVA immunization

100μg NP_{18}-OVA (Bioresearch Technologies) was suspended in a 1:1 H37RA CFA emulsion (Sigma) and injected subcutaneously into the left and right flanks. Seven days later mice were euthanized and inguinal draining lymph nodes (dLN) and/or spleen were harvested for flow cytometric analysis or suppression assays (see below).

In vitro T_{FR} suppression assay

The in vitro suppression assay was performed as previously reported (20, 21). Briefly, cell populations were sorted to 99% purity on an Aria II flow cytometer. Sorted cells were counted on an Accuri cytometer (BD Biosciences) by gating live cells only. 5×10^4 B cells, 3×10^4 T_{FH} cells (stained by Cell Trace Violet (Invitrogen) in indicated experiments according to the manufacturer's instructions) with or without 1.5×10^4 T_{FR} from dLNs were cultured with 2 μg/ml soluble anti-CD3 (2C11, BioXcell) and 5 μg/ml anti-IgM (Jackson Immunoresearch). After 6 days, cells were harvested and analyzed by flow cytometry. The proliferation of T_{FH} cells was detected by Ki67 staining or the dilution of Cell Trace Violet.

Statistical Analysis

Comparison of means between groups used the two-tailed Student's t-test. Differences were considered statistically significant at $P < 0.05$.

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Results

Enhanced humoral immune response in autoimmune CD28- Treg mice

CD28$^{fl/fl}$ mice were bred with Foxp3$^{YFP\text{-}Cre}$ mice to generate mice with a specific deletion of CD28 in Foxp3^{+} Tregs (CD28- Treg mice) (9). As previously reported, from birth to ~1 month of age, CD28- Treg mice had a relatively normal appearance. However by 8 to 12 weeks of age, CD28- Treg mice developed signs of autoimmunity. This was manifest as crusting eyelids, hair loss from the face and trunk, inflammatory infiltrates of the skin and lungs, and generalized lymphadenopathy and splenomegaly characterized by accumulation of activated (CD44^{hi}CD62L^{lo}) CD4^{+} and CD8^{+} T cells. These findings clearly implicated a Treg cell intrinsic function for CD28 in normal immune homeostasis, but did not define the role of individual immune effector components, i.e., T and/or B cells. The role of the latter was of particular interest, given the recent discovery of T_{FR} cells and their crucial role in controlling humoral immune responses. Thus we examined the role of B cells in the autoimmune phenotype of CD28- Treg mice.

Immunohistochemical staining revealed elevated numbers of B220+ B cells with larger follicle sizes, and enhanced IgG production in the follicles of enlarged LNs of 4 month-old CD28- Treg mice (Figure 1A–C). As CD28 is only deleted in mature Foxp3^{+} Tregs, we speculated that the abnormal B cell activation in CD28- Treg mice might be attributed to a defect of T_{FR} cells in the absence of CD28 signaling. The percentage of B cells in lymph nodes, but not spleen, was increased in inflamed 2 month-old CD28- Treg mice (Fig. 2A/B). Because of the increased total cellularity in the draining LNs of CD28- Treg mice, as well as the relatively normal percentages of most other immune cells such as NK cells, myeloid cells, and DCs (9), the enhanced B cell percentages and numbers indicate an actual and specific expansion of B cells (Fig. 2B). Concurrent with this, the serum IgG1 and IgG2a levels were increased in autoimmune CD28- Treg mice (Fig. 2C), although we did not observe any alterations in anti-double-stranded DNA antibodies (Fig. 2D). Nevertheless, germinal center B cells (GL-7^{+} Fas^{+}) were increased in CD28- Treg mice, along with increases in class-switched IgG^{+}IgM^{-}, IgG^{+}CD38^{+} effector/memory B cells, and plasma cells (Figure 2E–I). Finally, we noted evidence of general B cell activation, as B cells in CD28- Treg mice upregulated their surface expression of CD69, CD80 and MHCII (Figure 2J). Collectively, these data indicate that CD28-deficient mice have increased increase in B cell responses.

CD28 is required for the differentiation of follicular regulatory T cells

CD4^{+} T_{FH} cells migrate to B cell follicles to provide signals for B cell activation and antibody production (22, 23), while T_{FR} cells are the major regulatory population that control T_{FH} and B cell activation. Compared to control unimmunized wild type mice (CD28$^{+/+}$Foxp3-$^{YFP\text{-}Cre}$ mice), the lymph nodes of 2 month-old CD28- Treg mice showed significantly enhanced T_{FH} cell (CD4+Foxp3-CXCR5+ICOS+) percentages and numbers, with a reciprocal, but non-significant reduction of T_{FR} percentages (Figure 3A), suggesting that the reduced T_{FR} cell percentage and/or T_{FR} cell function in CD28- Treg mice results in a failure to regulate T_{FH} cell homeostasis. The low percentage of T_{FR} cells in the control conditions without immunization prevented a robust comparison of T_{FR} cell differentiation.

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However, the ratio of T_{FR} to T_{FH} cells is known to be a decisive factor in the magnitude of humoral immune response (10, 20), and the reduced T_{FR} to T_{FH} cell ratio in the CD28- Treg points to dysregulation of humoral immune responses. Thus, to further test the role of CD28 in T_{FR} cell differentiation, WT or CD28- Treg mice were immunized by NP-OVA/CFA subcutaneously. As shown in Figure 3B, NP-OVA immunization induced expansion of both T_{FH} and T_{FR} cells in WT mice, but CD28- Treg mice showed defective induction of T_{FR} cells in the draining lymph node.

As CD28- Treg have an autoimmune phenotype, it remained possible that the resultant inflammation was the cause of defective T_{FR} cell development, rather than due to a cell-intrinsic role for CD28 in T_{FR} cells. To address this question, we used female mice that were heterozygous for Foxp3-Cre (Foxp3$^{+/Cre}$). As shown previously by our group (9 and data not shown), due to random X-chromosome inactivation roughly 50% of Tregs in CD28$^{fl/fl}$ × Foxp3$^{+/Cre}$ mice have deleted CD28 (and can be identified by YFP expression) while half retain CD28 and are YFP^{-}. As shown in Figure 3C and 3D, we observed significantly higher percentages of CD28^{+} T_{FR} cells compared to CD28^{-} T_{FR} cells in both naïve and immunized mice. This is consistent with the data in Figure 3B showing lower numbers of T_{FR} cells in CD28- Treg mice and confirms a cell intrinsic role for CD28 in T_{FR} cell development.

Specific cytokines, such as IL-4 and IL-21 are important in supporting humoral responses. Thus we next examined selected cytokine production in CD28- Treg mice. Compared to WT control mice, we observed significantly elevated percentages of CD4^{+} cells from 3 month-old CD28- Treg mice secreting IL-4, and IL-21, and to a lesser extent IL-17 and IFNγ (Figure 3E and 3F). As IL-21 is the major cytokine to direct the generation of T_{FH} cells, its enhanced expression in CD28- Treg mice matched the increased humoral immune response (24, 25). In addition, B cells and IL-21 can activate and enhance the cytotoxicity of CD8^{+} cells (26, 27). Likewise, CD8^{+} cells in CD28- Treg mice demonstrated enhanced IFNγ production (Figure 3G and 3H).

CD28 is required for full suppressive function of follicular regulatory T cells

Previously, we demonstrated that global CD28-/- mice had severely reduced percentages of T_{FR} cells (20). However, here we found that the reduction in T_{FR} cells with conditional deletion of CD28 on Tregs was less profound, despite the marked failure to regulate antibody production. Therefore, we next determined whether loss of CD28 alters T_{FR} suppressive capacity. To do this, we performed an in vitro T_{FR} suppression assay in which WT (CD28$^{fl/+}$ X Foxp3$^{YFP\text{-}Cre}$) and CD28- Treg mice were immunized with NP-OVA. After 7 days, T_{FH} cells and B cells were sorted from control mice and were co-cultured in the presence or absence of T_{FR} cells from WT or CD28- Treg mice along with anti-IgM and anti-CD3 for 6 days. We found that CD28- T_{FR} cells suppressed the expression of Ki67, a cell cycle protein, and proliferation (Fig 4B) in T_{FH} cells to a much lesser degree compared to control T_{FR} cells (Figure 4A). Moreover, T_{FR} cells from CD28- Treg mice were less effective at suppressing class switch recombination and proliferation in B cells compared to control T_{FR} cells, suggesting that CD28 costimulation is essential for the full suppressive capacity of T_{FR} cells (Figure 4B, and 4C). Consistent with previous studies (28, 29), CXCR5^{-}ICOS^{-} Treg cells from immunized wild-type (CD28$^{fl/+}$ X Foxp3$^{YFP\text{-}Cre}$) mice were

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far less efficient at suppressing class switch recombination than were wild type T_{FR} cells, showing the same modest level of suppression as was observed for CD28-deleted T_{FR} cells (Figure 4D).

Previously, we have shown that CD28-deficient Tregs had reduced expression of CTLA-4 (9), a molecule known to regulate the proliferation and function of T_{FR} cells (30, 31). However, as shown in figure 4F, CD28- T_{FR} cells express comparable levels of CTLA-4, CD44, CD25 and ICOS as WT T_{FR} cells. Therefore, alterations in CTLA-4 did not appear to be to be responsible for the reduced suppressive capacity of CD28-deficient T_{FR}. We have also shown that ICOS expression on T_{FR} cells can be used to identify T_{FR} cells that gain access to the B cell follicle (29). We did not observe a difference in ICOS expression in CD28-deficient T_{FR} cells (Figure 4F). Using female mice that were heterozygous for Foxp3-Cre (Figure 4E, see details of experimental system in Figure 3), we observed diminished CXCR5 staining in CD28-deficient Treg cells, with little or no difference in bcl-6 expression. These data show a cell intrinsic role for CD28 in CXCR5 expression but suggest little role for CD28 signals in initiation of lineage specific transcription factor expression.

B cells are required for the autoimmunity in CD28- Treg mice

To assess whether or not the skin autoimmunity in CD28- Treg mice was due to enhanced B cell responses allowed by defective T_{FR} cell differentiation and function, we bred CD28- Treg mice to μMT mice, which lack B cells (19). As shown in supplementary figure 1, and consistent with previous studies (32, 33), loss of B cells led to a block of T_{FH} cell differentiation in CD28- Treg × μMT mice. Strikingly, the lack of B cells rescued the severe autoimmunity seen in CD28- Treg mice. This included the phenotype of grossly visible skin inflammation (Figure 5A, indicated by arrows), lymphoadenopathy (Figure 5B), and inflammatory histology in the skin and lung, the main target organs of disease we observed in CD28- Treg mice (9) (Figure 5C).

Finally, we examined T cell activation in lymphoid organs of CD28- Treg × μMT mice. B cell deficiency completely prevented the activation of $CD4^+$ T cells, but not $CD8^+$ T cells in the lymph nodes and spleen of CD28- Treg × μMT mice (Figure 6A and 6B and Supplementary Figure 2). Interestingly, although $CD8^+$ effectors were not prevented, IFNγ production by both $CD4^+$ and $CD8^+$ T cells was reduced (Figure 6C and 6D). Collectively, these results indicate a dissociation between the role of B cells in mediating $CD4^+$ vs. $CD8^+$ T cell activation in CD28- Treg mice, and demonstrate that B cells are required for activation of $CD4^+$ T cells and/or antibody production for the autoimmunity observed in these animals.

Discussion

Previously we reported that cell-specific deletion of CD28 on $Foxp3^+$ regulatory T cells (CD28- Treg mice) led to a systemic autoimmune disease characterized by activation of $CD4^+$ and $CD8^+$ T cells, with skin and lung inflammation (9). Here, we further characterized these animals and found a striking elevation of serum immunoglobulins accompanied by increases in IgG class switched and memory B cells, as well as germinal center B cells in the lymph nodes of these animals.

It is well established that B cell activation in GCs is regulated by T_{FR} cells. This prompted us to examine how loss of CD28 in Tregs might specifically affect T_{FR} cells. We found a marked reduction in T_{FR} cells, both in unimmunized animals, and following immunization. This suggested that the dysregulation of B cell responses observed in unmanipulated CD28-Treg mice might be due to alterations in T_{FR} numbers. However another, non-mutually exclusive possibility was that optimal T_{FR} function required CD28 and thus the ability of T_{FR} cells to control humoral responses was intrinsically defective, independent of cell number. To test this possibility, we turned to an in vitro assay of T_{FR} cell suppressive capability, and showed that indeed, loss of CD28 led to an impairment in T_{FR} cell function.

The mechanism by which CD28 is required for optimal T_{FR} cell function remains under investigation. Previously, CD28 signaling in $CD4^+$ T cells has been shown to induce the expression of both CXCR5 and ICOS (34, 35). Compared to WT T_{FR}, CD28- T_{reg} cells did not demonstrate a significant reduction in the expression of ICOS. However, it is still plausible that CD28 signaling may regulate the coexpression of CXCR5 and ICOS in T_{reg} cells that might dictate T_{FR} cell induction upon immune stimulation.

Perhaps the most striking finding in our studies was the loss of aberrant CD4+ T cell activation and the prevention of "clinical" autoimmunity by genetic deletion of B cells, indicating a strong and unexpected B cell contribution to autoimmunity. Interestingly, previous work has demonstrated that the absence of B cells, or therapeutic depletion of B cells, reduces autoimmune pathology and prolongs survival in scurfy mice (36). In a related vein, a number of clinical trials have shown that B cell depletional therapies ameliorate autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis, which are believed to have predominant, if not exclusive, T cell pathogenesis (37–39). Collectively, our findings demonstrate a clear role for B cells in T cell-driven autoimmune inflammation. It seems likely that this role is via antigen presentation, although this remains to be formally demonstrated and other factors such as antibody or cytokine production cannot be completely excluded.

Interestingly, despite the fact that loss of B cells prevented overt autoimmunity from developing, we found a "split" effect on T cell activation. Specifically, while B cell depletion prevented the activation of $CD4^+$ T cells, there was little to no effect on $CD8^+$ T cell activation. This may indicate a differential/preferential reliance of $CD4^+$ vs $CD8^+$ T cells for types of APCs. Alternatively, the functional deficiency of CD28- Tregs may induce aberrant activation of CD8+ T cells.

In summary, our data reveal an essential role for CD28 in directing the development and function of T_{FR} cells to control B cell activation and humoral immune responses. In the autoimmune disease model of CD28- Treg mice, reduction in T_{FR} cell differentiation and suppressive function results in broad dysregulation of B cell homeostasis. Importantly, genetic depletion of B cells in CD28- Treg mice prevented $CD4^+$ T cell activation and rescued the autoimmune phenotype. Although other Treg subsets may contribute, our findings suggest that alterations in T_{FR} cell differentiation and/or function results in systemic autoimmunity and skin inflammation. Future work will be needed to determine

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how promoting T_{FR} cell differentiation and function can be used to help treat autoimmune diseases including psoriasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Footnotes:

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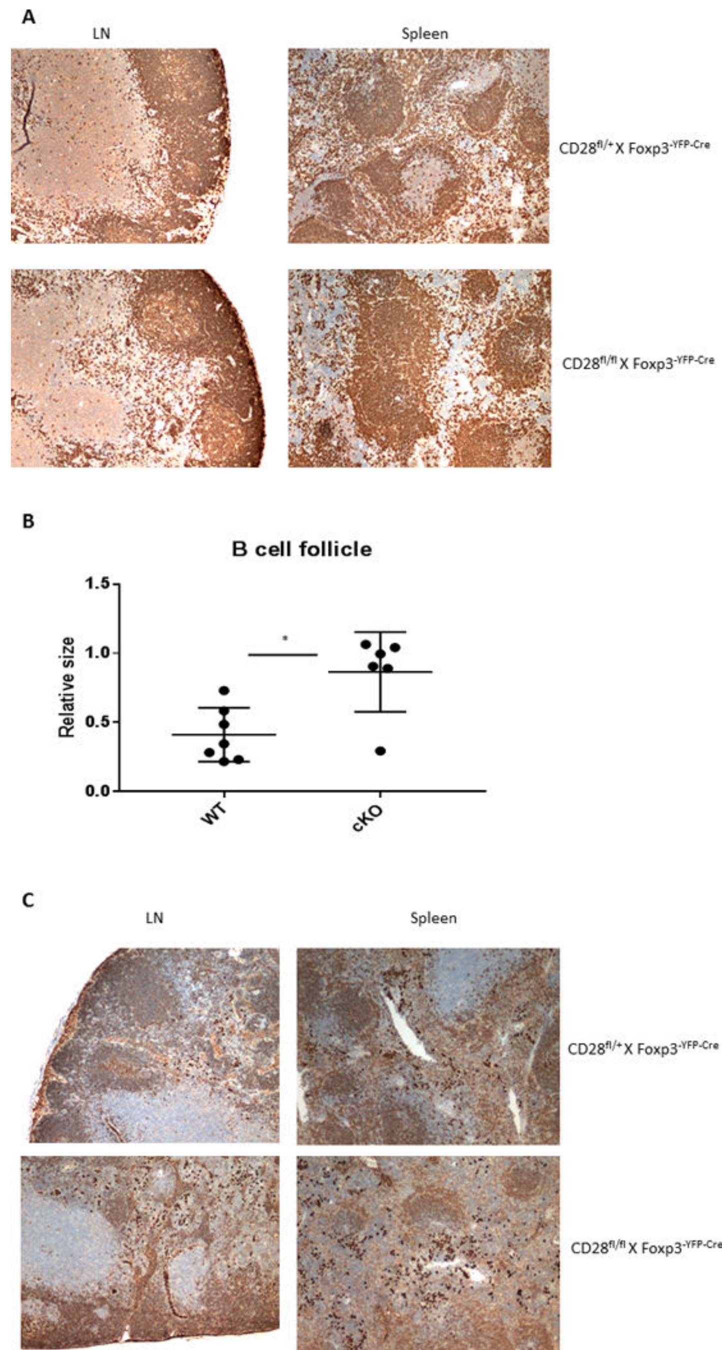

Figure 1.

B cell and IgG production in LN and spleen of CD28- Treg mice. 4 month-old autoimmune CD28- Treg mice were sacrificed and LNs and spleens were fixed in 10% formalin overnight. Immunohistochemistry was performed to detect B cell (B220 staining) in **A** and follicle sizes were measured by Image J in **B**. IgG production is shown in **C**. WT: wild type mice. cKO: CD28- Treg mice. *, P<0.05. Magnification: 100X. Minimum of two animals in each phenotype were analyzed.

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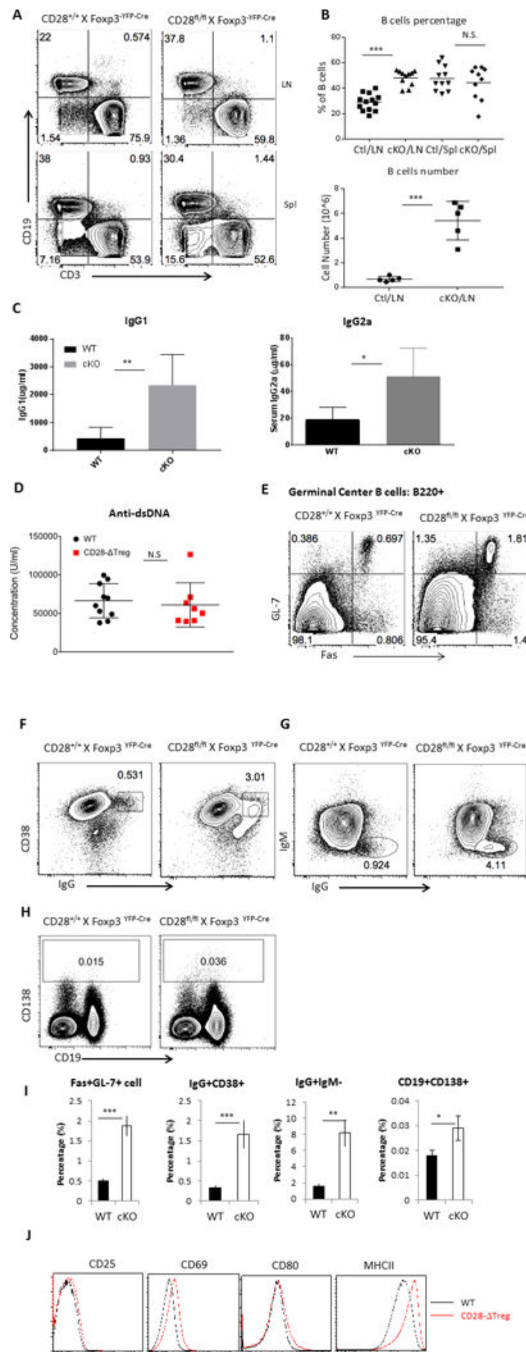

Figure 2.

B cell expansion and activation in CD28- Treg mice. **A**. Representative plot of B cells in LNs of 2-3 months old WT and CD28- Treg mice. **B**. Summary of A. Each dot represents one mouse. **C/D**. Serum IgG1 and IgG2a (**C**) and anti-dsDNA antibodies (**D**) in 2~3 month old mice. **E/F/G/H**. Characterization of B cells in LNs. Gated B220$^+$ or CD19$^+$ B cells were stained for Fas+GL7+ germinal center cells (**E**), IgG$^+$CD38$^+$ cells (**F**), IgG$^+$IgM$^-$ (**G**), and CD19$^+$CD138$^+$ plasma cells (**H**). (**I**). Statistical analysis. **J**. Representative surface expression of CD25, CD80, CD69 and MHCII in B cells (B220+) of 2 month old WT of

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CD28- Treg mice. *, P<0.05. **, P<0.01. ***, P<0.001. N.S., not significant. Over 5 WT and 5 CD28- Treg mice were analyzed.

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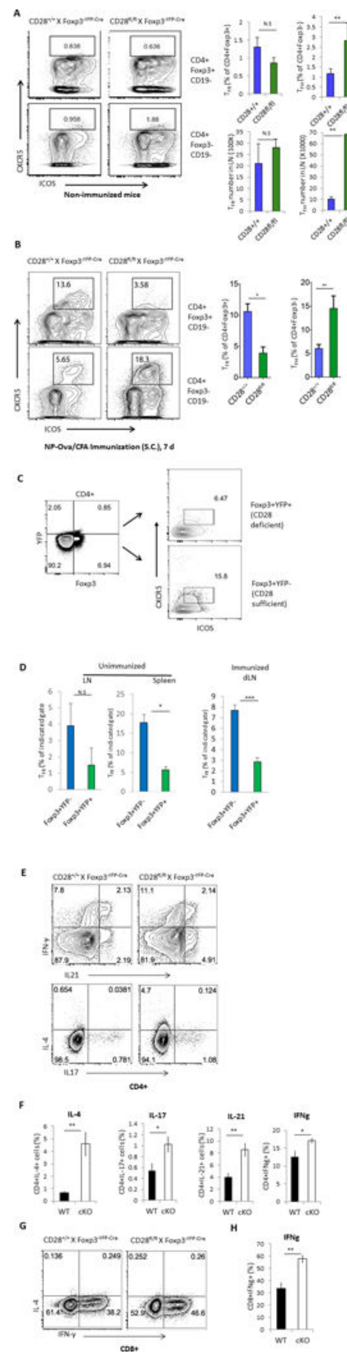

Figure 3.

CD28 is required for T_{FR} cell differentiation in LNs. **A**. Percentage and number of T_{FH} in total $CD4^+YFP^-$ cells and percentage and number of T_{FR} in $CD4^+YFP^+$ Tregs in LNs of WT and CD28- Treg mice (YFP is a Foxp3 reporter). **B**. WT and CD28- Treg mice were subcutaneously immunized with NP-OVA/CFA. 7 days later, T_{FH} and T_{FR} percentage were analyzed as A. Representative flow cytometry plots (left) and quantification (right). **C/D**. In healthy $CD28^{fl/fl}$ X Foxp3-$^{YFP\text{-}CRE/+}$ female mice (unimmunized or NP-OVA-immunized), CD28-deficient T_{FR} ($CD4^+Foxp3^+YFP^+CXCR5^+ICOS^+$) and CD28-sufficient T_{FR}

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($CD4^{+}Foxp3^{+}YFP^{-}CXCR5^{+}ICOS^{+}$) were gated as C (representative of immunized dLN) and the percentage of T_{FR} were shown in **D**. 2 to 3 animals were analyzed. **E/F/G/H**. Splenocytes of WT and CD28- Treg mice were stimulated in vitro with PMA and Ionomycin for 5 hours in the presence of Golgi Block. Representative flow cytometry of analyses of IL-4, IL-17, IL-21 and IFNγ in $CD4^{+}$ cells (E) and IFNγ in $CD8^{+}$ cells (G) and their quantification (F and H) are shown. *, P<0.05. **, P<0.01. ***, P<0.001. Data are representative of over 4 WT and 4 CD28- Treg mice analyzed.

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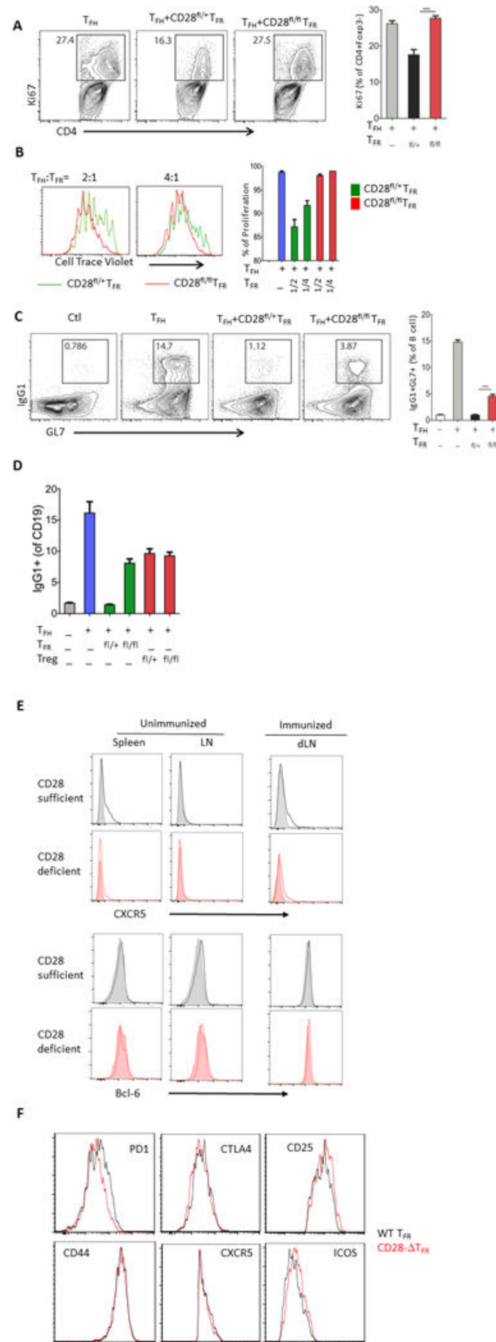

Figure 4.

CD28 is required for the suppressive function of T_{FR} cells. **A/B/C/D/E**. Sorted GL7^{-} B cells and CD4^{+}Foxp3^{-} T_{FH} cells were co-cultured with CD28-sufficient T_{FR} (CD28$^{fl/+}$$T_{FR}$ from CD28$^{fl/+}$ × Foxp3$^{YFP\text{-}Cre}$ mice) or CD28-deficient T_{FR} (CD28$^{fl/fl}$$T_{FR}$ from CD28$^{fl/fl}$ × Foxp3$^{YFP\text{-}Cre}$ mice) for 6 days in vitro. The proliferation of T_{FH} cells were detected by Ki67 staining and Cell Trace violet dilution (**A and B**). In **C**, B cells were stained for GL7+IgG1+. **D**. Similar as C, CD28-sufficient Tregs (CD28$^{fl/+}$ × Foxp3$^{YFP\text{-}Cre}$) and CD28-deficient Tregs (CD28$^{fl/fl}$ X Foxp3$^{YFP\text{-}Cre}$) were also sorted and co-cultured with T_{FH} and B

cells in the suppression assay. **E**. CD28 sufficient ($CD4^+Foxp3^+YFP^-$) and CD28 deficient Tregs ($CD4^+Foxp3^+YFP^+$) from LN or spleen of healthy $CD28^{fl/fl}$ X $Foxp3^{YFP-Cre/+}$ female mice were compared for CXCR5 and Bcl6 expression. Filled line is isotype staining. **F**. Treg markers in WT and CD28-deficient T_{FR} from 3 months old WT and autoimmune CD28-
Treg mice. ***, P<0.001. Experiments were repeated at least four times.

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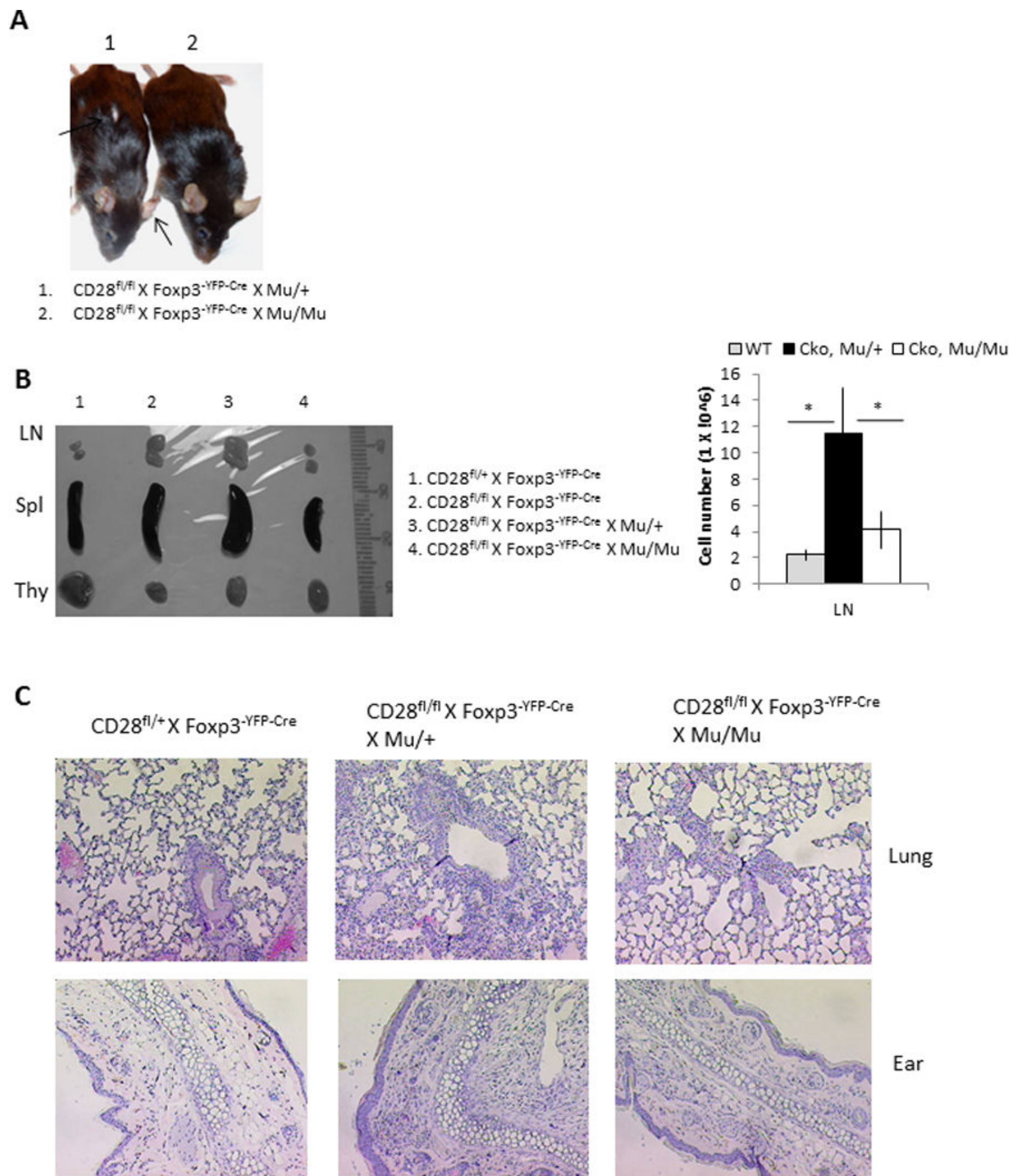

Figure 5.

B cell deficiency prevents autoimmunity in CD28- Treg mice. **A**. Autoimmune phenotype in 5 month-old CD28$^{fl/fl}$ X Foxp3$^{YFP-Cre}$ mice is abrogated in CD28$^{fl/fl}$X Foxp3$^{YFP-Cre}$X Mu/Mu. Arrows indicate inflammation. **B**. Macroscopic appearance of LN, spleen and thymus in CD28$^{fl/+}$ X Foxp3$^{YFP-Cre}$ (1), CD28$^{fl/fl}$ X Foxp3$^{YFP-Cre}$ (2), CD28$^{fl/fl}$ X Foxp3$^{YFP-Cre}$ X Mu/+(3), and CD28$^{fl/fl}$ X Foxp3$^{YFP-Cre}$ X Mu/MuT (4). **C**. H&E staining of fixed lung and ear tissues from 5 months old CD28$^{fl/+}$ X Foxp3$^{YFP-Cre}$, CD28$^{fl/fl}$ X

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$Foxp3^{YFP\text{-}Cre}$ X Mu/+, and $CD28^{fl/fl}$ X $Foxp3^{YFP\text{-}Cre}$ X Mu/Mu. Magnification: 100X. *, P<0.05. Data are representative of over 3 animals in each phenotype analyzed.

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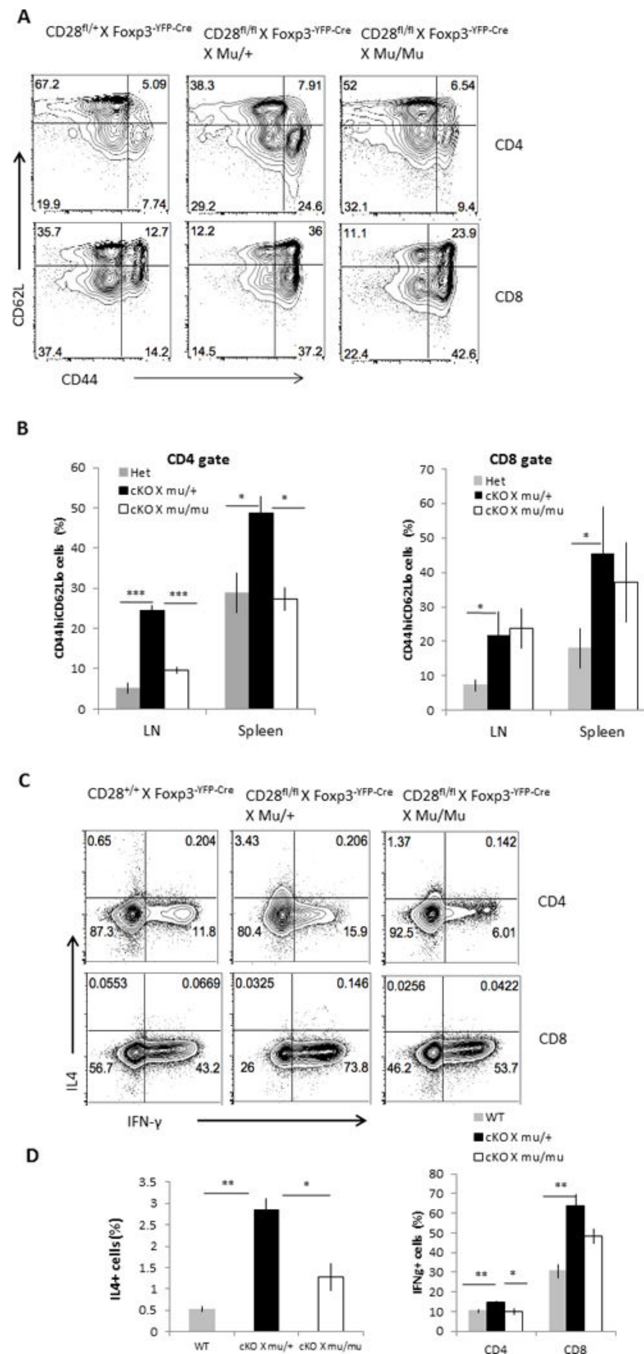

Figure 6.

B cells deficiency prevents over-activation of CD4+ T cells in CD28- Treg mice. Representative flow cytometry plots (**A**) and quantification (**B**). Percentage of $CD44^{hi}CD62L^{lo}$ effector cells in $CD4^+$ and $CD8^+$ T cells from LNs of 5 month old $CD28^{fl/fl}$ X $Foxp3^{YFP\text{-}Cre}$ X Mu/+ and $CD28^{fl/fl}$ X $Foxp3^{YFP\text{-}Cre}$ X Mu/Mu mice. Representative flow cytometry plots (**C**) and quantification (**D**). IL-4 production in $CD4^+$ splenic T cells and IFNγ production in $CD4^+$ and $CD8^+$ splenic T cells of 5 months old mice. Splenocytes were stimulated in vitro by PMA and Ionomycin for 5 hours in the presence of Golgi block. WT:

wild type mice. cKO X Mu/+: $CD28^{fl/fl}$ X $Foxp3^{YFP\text{-}Cre}$ X Mu/+ mice. cKO X Mu/Mu: $CD28^{fl/fl}$ X $Foxp3^{YFP\text{-}Cre}$ X Mu/Mu mice. *, P<0.05. **, P<0.01. ***, P<0.001. Over 3 animals in each phenotype were analyzed.

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