Foxp3+ Regulatory T Cells Control Humoral Autoimmunity by Suppressing the Development of Long-Lived Plasma Cells

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Foxp3\(^+\) Regulatory T Cells Control Humoral Autoimmunity by Suppressing the Development of Long-Lived Plasma Cells

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Foxp3\(^+\) regulatory T cells (Tregs) are crucial for maintaining T cell tolerance, but their role in humoral autoimmunity remains unclear. To address this, we combined a model of autoantibody-dependent arthritis (K/BxN) with Foxp3 mutant scurfy mice to generate Treg-deficient K/BxN mice, referred to as K/BxNsf mice. The disease symptoms of K/BxNsf mice were exacerbated, and this coincided with increases in extrafollicular Th cells, follicular Th cells, and germinal centers. Surprisingly, the K/BxNsf mice exhibited an abnormal accumulation of mature plasma cells in their spleens and a corresponding loss of bone marrow plasma cells. The plasma cells were unresponsive to the bone marrow homing chemokine CXCL12, despite normal expression of the chemokine receptor CXCR4. Importantly, they were long-lived and less susceptible to the cytotoxic action of cyclophosphamide. They also expressed less Fc\(\gamma RIIb\) and were less apoptotic in response to autoantigen-autoantibody immune complexes. This suggests that Tregs control plasma cell susceptibility to cell death induced by engagement of Fc\(\gamma RIIb\) with immune complexes. Direct cytotoxic effects of Tregs also contribute to the death of plasma cells. Thus, our results reveal that Tregs suppress the emergence of long-lived splenic plasma cells by affecting plasma cell-autonomous mechanisms as well as T cell help, thereby avoiding the persistence of humoral autoimmunity. *The Journal of Immunology, 2011, 186: 000–000.

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ystemic autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis, are characterized by activation of autoreactive T and B cells (1, 2). This activation represents a functional loss of self-tolerance and leads to autoantibody production. Autoantibodies trigger complement- and FcR-mediated inflammatory responses in the target tissue, which in turn promote tissue destruction, more extensive loss of self-tolerance, and ultimately symptoms of autoimmunity (3). Therefore, persistent production of pathogenic autoantibodies is central to the chronic, destructive clinical manifestations of systemic autoimmune diseases.

The persistence of autoantibodies in autoimmune disorders can be attributed either to a continuous supply of short-lived plasma blasts or to the activity of long-lived plasma cells (PLCs). Both of these cell populations develop as a result of Ag-specific, cognate interactions with extrafollicular T helper (TEFH) cells at extrafollicular B cell foci or with follicular T helper (TFH) cells within follicular B cell foci, respectively (3, 4). Therefore, persistent production of pathogenic autoantibodies is central to the chronic, destructive clinical manifestations of systemic autoimmune diseases. The persistence of autoantibodies in autoimmune disorders can be attributed either to a continuous supply of short-lived plasma blasts or to the activity of long-lived plasma cells (PLCs). Both of these cell populations develop as a result of Ag-specific, cognate interactions with extrafollicular T helper (TEFH) cells at extrafollicular B cell foci or with follicular T helper (TFH) cells within follicular B cell foci, respectively (3, 4). Therefore, persistent production of pathogenic autoantibodies is central to the chronic, destructive clinical manifestations of systemic autoimmune diseases.

The APCs that emerge from these reactions are dividing, rapidly turning over, Ab-secreting plasmablasts, which in turn differentiate into nondoning PCs (6). Most PCs either die within 3–4 d in these organs, which apparently provide only few survival niches for the cells, or leave the organs in search of survival niches provided mainly by the bone marrow (BM). After arriving at the BM, PCs terminally differentiate to end-stage Ab-secreting cells and attain a long life span of months or even years. Therefore, although a small proportion of long-lived PCs persist in the spleen and LNs, most long-lived PCs reside in the BM. These PCs are not eliminated by treatments targeting B lineage cells, such as irradiation, prednisone, cyclophosphamide, and anti-CD20 Abs (7–10). Thus, along with their persistent Ab production, the resistance of these PCs to cytostatic treatment contributes to the difficulty of resolving long-lived PC-mediated diseases. Despite the pathological significance of long-lived PCs, little is known about how they develop and persist in vivo in autoimmune states.

Several of the steps generating peripheral autoimmunity can be censored by CD4\(^+\)Foxp3\(^+\) regulatory T cells (Tregs). These cells were originally identified by their capacity to suppress the proliferation and activation of other T cells (11). However, recent studies have extended their targets to diverse immune cells including B cells. Tregs have been shown to suppress directly the activation of B cells, in addition to their indirect effect through suppressing the activity of TFH cells (12, 13). Their suppressive activities include perforin/granzyme-dependent cytotoxicity targeting B cells as well as T cells (14–16). In agreement with these in vitro studies, the titer of autoantibodies in autoimmune subjects is inversely correlated with the activity of Tregs. For instance, depletion and transfer of Tregs in autoimmune animals lead to increased and decreased autoantibody production, respectively (17, 18). Reversal of the numerical deficit of Tregs in SLE reduces titers of autoantibodies in vivo (19). However, how Tregs affect the activity of autoantibody-producing PCs in vivo remains unknown.

We undertook the current study to investigate whether Tregs alter the physiology of PCs, using a murine autoantibody-mediated

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; BM, bone marrow; dLN, joint-draining lymph node; GC, germinal center; GPL, glucose-6-phosphate isomerase; LN, lymph node; PC, plasma cell; PSGL-1, P-selectin glycoprotein ligand 1; SLE, systemic lupus erythematosus; T\(_{\text{Efh}}\), extrafollicular helper T; T\(_{\text{Ffh}}\), follicular helper T; Treg, regulatory T cell.

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disease model named K/BxN. The spontaneous development of severe arthritis in this model depends on the production of autoantibodies to glucose-6-phosphate isomerase (GPI) (20). Previously, we found that despite the loss of self-tolerance, functionally intact Foxp3+ Tregs exist in the K/BxN mice (21). This prompted us to consider the in vivo role of Tregs in autoantibody production. We show in this study that in addition to enhancing the development of Th cell subsets, Treg deficiency results in changes intrinsic to PCs—as evidenced by their aberrant localization, maturation, and life span—which favor the persistence of humoral memory. Thus, our results demonstrating PC regulation by Tregs provide novel insight into how Tregs limit humoral memory.

Materials and Methods

Mice

A cross between KRJ TCR-transgenic mice on a C57BL/6 background (K/B) and NOD mice generated arthritic transgenic progeny (K/BxN) and nontransgenic littermates (BxN) (20). Female C57BL/6 mice bearing the scurfy (sf) allele (The Jackson Laboratory) were back-crossed to NOD for more than six generations to generate scurfy carrier female mice on an NOD background (NODsf). NODsf female mice were crossed with K/B to generate male K/BxN mice bearing the sf allele (referred to as K/BxNsf mice hereafter). All mice were maintained in a specific pathogen-free barrier facility at Hanyang University. The study was approved by the institutional animal care and use committee.

Cell culture

To determine whether Tregs are cytotoxic to PCs, coculture experiments were carried out. CD4+CD25+ and B220+ cells from the spleens of C57BL/6 mice were purified by MACS (Miltenyi Biotec). Each cell fraction was more than 97% pure. CD4+CD25+ cells were preactivated with 5 μg/ml anti-CD3 mAb, 1 μg/ml anti-CD28 mAb, and 100 U/ml IL-2 (all from BD Bioscience) for 2 d. B220+ cells were stimulated with 10 μg/ml LPS for 2 d, washed, and cocultured with equal numbers of preactivated CD4+CD25+ cells in the presence of 1 μg/ml anti-CD3 mAb, 1 μg/ml anti-CD28 mAb, 100 U/ml IL-2, and 1 μg/ml LPS, followed by FACS analyses. To determine whether Tregs affect FcγRII expression in PCs, naive Tregs purified from BxN mice were cocultured for 24 h with PCs that had been formed in vitro as above or purified from K/BxN mice by MACS. The cells were then analyzed by FACS. To determine whether PCs are cytotoxic for immune complexes, FcγR immune complexes were made by adding 10 μg/ml FcγRIIa to serum from 8-wk-old K/BxN mice at a ratio of 1:1 (v/v). Normal serum from BxN mice served as a negative control. B220+CD138+ PCs purified by MACS were incubated with the experimental and control sera for 9 h, stained with allophycocyanin-conjugated annexin V and 7-AAD, and assayed by FACS.

Results

Disease exacerbation in K/BxNsf mice is associated with enhanced activation of both extrafollicular and follicular pathways of Ab responses

To investigate the in vivo role of Tregs in humoral autoimmunity, we generated Treg-deficient K/BxNsfs mice by congenically combining the K/BxN mice with Foxp3 mutant scurfy mice. We found that K/BxNsfs mice exhibited earlier onset and more aggressive progression of arthritis than their K/BxN littermates, consistent with previous findings (24). In particular, titers of serum Abs against GPI were detectable from 4 wk and peaked at ~8 wk in K/BxNsfs mice, which were 10–14 d earlier than in K/BxN mice (data not shown).

T cell-dependent Ab responses can take place in either extrafollicular or follicular areas. These two independent responses are governed by two distinct populations of Th cells, CD4+CD45RbCD62LloPSG-1lo TEFH cells and CD4+CD25+CXCR5+ICOS+ TFH cells (4, 5). Both populations emerged in the spleen and dLNs of K/BxN mice and gradually expanded with age, suggesting that both extrafollicular and follicular pathways of Ab responses are involved in the pathology of K/BxN mice (Fig. 1A, 1B). K/BxNsfs mice contained much larger numbers of TEFH and TFH cells in their spleens and dLNs than those of K/BxN mice. These data suggest that Tregs limit the development of TEFH and TFH cells in vivo, ultimately regulating B cell activation.

In the central, follicular dendritic cell-rich region of the follicle, TFH cells help Ag-engaged B cells to form GCs. Because post-GC B cells give rise to isotype-switched, high-affinity clones and leave the GC as PCs and memory B cells, this GC-dependent pathway of B cell differentiation is crucial for long-term humoral
responses (25). We found that K/BxNsf mice developed GCs ~2 wk earlier and in greater numbers than K/BxN mice (Fig. 1C, 1D). Along with data demonstrating the presence of Foxp3+ Tregs within the GC (Fig. 1E), these results provide robust evidence that Tregs affect the kinetics of GC formation and quantity of GCs in vivo.

**Treg deficiency results in aberrant accumulation of mature PCs in the spleen and LNs of K/BxN mice**

We tested whether the loss of Tregs affects the behavior of Ab-secreting PCs. PCs formed in the K/BxN mice resembled those developing upon immunization with foreign protein Ags in several ways. First, spleen and dLNs from K/BxN mice contained a small but significantly larger fraction of CD138+ PCs than that from normal BxN mice and consisted predominantly of CD138+B220hi early PCs (plasmablasts) rather than CD138+B220lo mature PCs (Fig. 2A, 2B). Second, a substantial fraction of autoantibody-secreting PCs resided in the BM (Fig. 2C). In contrast, K/BxNsf mice contained a substantially higher fraction and number of B220loCD138+ mature PCs in their spleens and dLNs, despite a significantly lower number of total B220+ cells (Fig. 2A, 2B). This phenomenon was not seen in older K/BxN mice (10 wk old) with full-blown arthritis, suggesting it represents a qualitative change not simply a matter of kinetics. In ELISPOT assays measuring the frequency of autoantibody-secreting PCs, GPI-specific IgG1 Ab-secreting cells were ~5-fold more numerous in the spleen and ~3-fold less numerous in the BM of K/BxNsf mice than that in their K/BxN counterparts (Fig. 2C). Immunofluorescence staining further confirmed the abundance of PCs in the spleen, especially in the extrafollicular space of splenic red pulp (Fig. 2D). Taken together, our results demonstrate that Treg deficiency results in abnormal accumulation of mature PCs in the extrafollicular area of the spleen and dLNs with a concordant loss of BM PCs. This suggests that the splenic accumulation of PCs is due to failure of BM homing, or vice versa.

**Splenic PCs from K/BxNsf mice are unresponsive to CXCL12, despite expressing its receptor normally**

PC migration to the BM is largely controlled by an interaction between CXCL12 and its receptor CXCR4 (26). After activation within the GC, the expression of CXCR4 is enhanced on the surface of post-GC B cells. CXCL12 is produced by BM stromal cells and guides the recruitment of CXCR4+ cells to the BM. To address whether splenic PCs from K/BxNsf mice were responsive to CXCL12–induced migration, we carried out ex vivo chemotaxis assays. The vast majority of K/BxNsf PCs did not migrate toward CXCL12, whereas a large fraction of K/BxN mice were responsive to CXCL12–induced migration (Fig. 3A). Thus, PCs developing in a milieu lacking Tregs seem to lack the capacity to respond to CXCL12, and this affects their capacity for BM homing.

To determine whether this migratory defect resulted from reduced expression of CXCL12 receptor, we measured the level of cell surface CXCR4 on PCs by FACS analysis. Early (B220hi) and late (B220lo) PCs from K/BxNsf mice expressed even higher levels of CXCR4 than those from K/BxN mice (Fig. 3B). In addition, VLA-4, an adhesion molecule that is involved in the retention of PCs in the BM, was also enhanced on the surfaces of PCs from K/BxNsf mice. Thus, the defective responsiveness of PCs to CXCL12 in K/BxNsf mice is not due to lower expression of its receptor on the PC.

**Splenic PCs from K/BxNsf mice live longer and are less susceptible to cytostatic agents than those from K/BxN mice**

During normal T cell-dependent responses, most PCs residing in the spleen and LNs die within 3–4 d because these organs provide...
only few survival niches for the cells. To determine whether Tregs can affect the life span of PCs in addition to their migratory behavior, K/BxNsf and their counterpart littermates were fed BrdU for 14–28 d, and incorporation was assayed by FACS. Surprisingly, more than 95% of viable PCs from the spleens and dLNs of K/BxNsf mice survived for at least 28 d without cell division, whereas 80–90% of those from K/BxN mice divided and were short-lived (Fig. 4A).

A hallmark of long-lived PCs is their resistance to cytostatic treatment with immunosuppressants, such as cyclophosphamide (9). To determine whether splenic PCs from K/BxNsf mice were resistant to the cytotoxic effect of cyclophosphamide than are K/BxN PCs (Fig. 4B). Thus, these results demonstrate that Tregs act to reduce the development of cytotoxic drug-resistant, long-lived splenic PCs in vivo.

Tregs control the survival of PCs via direct and FcγRIb-mediated death mechanisms

It is well established that Tregs elicit the death of T and B cells in a perforin/granzyme-dependent manner (14–16, 27), but the existence of such an effect on PCs has not been explored. Our data showing Tregs in contact with splenic PCs in the extrafollicular area (Fig. 5A) suggest direct action of Tregs on PCs. Indeed, we found that CD138+ PCs formed in vitro underwent increased cell death, as evidenced by increased annexin V+7-AAD+ cell fraction.
and by decreased viable PC number when cocultured with Tregs in wells but not when separated from the Tregs in Transwells (Fig. 5B, 5C). Thus, these results demonstrate that Treg-mediated killing of PCs involves a contact-dependent mechanism.

Why are splenic PCs from K/BxNsF mice long-lived in an immune complex-rich milieu? This question arises because K/BxNsf mice contain large numbers of immune complexes (28), and PCs readily undergo apoptotic death when FcγRIIb molecules expressed on them are cross-linked with immune complexes (29). To answer this question, we first measured the level of FcγRIIb on the PCs and found that the surface level of FcγRIIb was significantly lower in the splenic PCs from K/BxNsF mice than in those from K/BxN mice or normal BxN mice (Fig. 6A). Accordingly, FcγRIIb transcripts were significantly reduced in both B220<sup>+</sup> cells as a whole and in B220<sup>+</sup>CD138<sup>+</sup> PCs from K/BxNsF mice (Fig. 6B). Therefore, these data demonstrate that the absence of Tregs results in downregulation of the death receptor FcγRIIb on PCs.

To determine whether the reduced FcγRIIb expression affected immune complex-mediated cell death of the K/BxNsF PCs, purified splenic PCs from K/BxN and K/BxNsF mice were incubated with a mixture of GPI and K/BxN serum containing anti-GPI Abs or a mixture of GPI and normal BxN serum as a control. The K/BxN PCs underwent greater apoptosis (annexin V<sup>+</sup>7-AAD<sup>+</sup>) in response to the GPI/K/BxN serum than to the GPI/BxN serum, demonstrating that cross-linking of FcγRIIb by immune complexes of GPI and anti-GPI Abs can induce PC apoptosis (Fig. 6C). In contrast, the GPI/BxN serum did not induce more apoptosis of the K/BxNsF PCs than did the GPI/BxN serum, demonstrating that the K/BxNsF PCs were not susceptible to the immune complex-mediated apoptosis that is dependent on the abundance of FcγRIIb.

The reduced FcγRIIb expression on K/BxNsF PCs could mean that Tregs directly upregulate FcγRIIb expression on PCs. To test this, we cocultured B220<sup>+</sup>CD138<sup>+</sup> PCs purified from K/BxN mice with syngeneic Tregs and found that FcγRIIb levels on the PCs were not influenced by the presence of Tregs (Fig. 6D). We obtained the same result with PCs generated in vitro (data not shown). Therefore, Tregs do not seem to directly signal PCs to induce FcγRIIb expression.

**Discussion**

Although Treg deficiency has been shown previously to exacerbate the disease symptoms in the K/BxN model (24), the mechanism by which Tregs elicit their effects on components of humoral autoimmunity remains to be explored. In the current study, we found that the absence of Tregs elicits quantitative and qualitative alterations in diverse components of humoral immunity. Our data showing more abundant numbers of T<sub>FEFH</sub> and T<sub>FH</sub> cells in K/BxNsF mice implicate Tregs in regulating the quantity of these cells in vivo. The abundance of T<sub>FH</sub> cells is accompanied by an increase in the quantity of GCs and PCs. The increase in PCs far surpasses B cell insufficiency, which presumably stems from abnormal lymphopoiesis in the BM, as reported recently in the scurfy model (30). Importantly, the most prominent qualitative influence of Tregs is on the physiology of PCs. Contrary to the behavior of normal PCs emerging in a Treg-replete milieu, PCs emerging in a milieu lacking Tregs are nonmigratory and long-lived. They survived at least for 4 wk without cell division, which fits the criterion for long-lived PCs based on previous studies using BrdU incorporation assays (31–34). However, because cellular markers used here are not necessarily markers of long-lived PCs, and because we did not distinguish individual PCs in each time point, the long-lived nature of PCs was only determined by their survival and cell division.
others have reported that long-lived PCs can live for several months (8, 9), it would be of interest to determine how long K/BxNsf PCs can actually survive in the spleen. Given the pathogenic characteristics of autoreactive long-lived PCs, our results suggest that the activity of Tregs is important for limiting the persistence of humoral immune responses.

Previous studies have pointed to various kinds of cross-talk between regulatory cells and TFH cells. In autoimmune animals, persistence of humoral immune responses is limited when regulatory cells and TFH cells are cocultured in wells or incubated separately in Transwells (Tw). FACS profiles gated on B220loCD138+ cells after 8 h culture are shown (B). Preactivated B220+ cells were cocultured with preactivated CD4+CD25+ cells in the presence of stimuli as described in Materials and Methods. The two populations were either cocultured in wells or incubated separately in Transwells (Tw). FACS profiles gated on B220+CD138+ cells after 8 h culture are shown (B). Viable B220+CD138+ cell numbers after 48 h culture are shown as percentages (C). The data are representative of three independent experiments. **p < 0.01 (Student t test).

We found that the K/BxNsf PCs, which accumulated in abnormal high numbers in the spleen and did not migrate into the BM, were unresponsive to CXCL12 in ex vivo chemotaxis assays despite the fact that they expressed normal levels of its receptor, CXCR4. Because CXCL12 is a major BM homing chemokine, this CXCL12 unresponsiveness may be responsible for the failure of the PCs to egress from the spleen. Our findings are in line with the observation that PC emigrants into the BM progressively lose their chemotactic responsiveness to CXCL12 as they become terminally mature and long-lived, even though they continue to express CXCR4 (37). It is conceivable that in the absence of Tregs, the PCs age unusually rapidly in the spleen, so that their responsiveness to CXCL12 is prematurely terminated. It should also be noted that PC unresponsiveness to CXCL12 does not necessarily result in BM homing failure, as it has been shown that PCs can home into the BM through a pathway independent of, albeit less efficient than, the CXCL12/CXCR4 axis (38). However, it is not likely that this alternative pathway is activated in the K/BxNsf PCs, because they were barely to be seen in the BM. It is also of interest that CXCR4 surface expression did not lead to CXCR4-induced migration. We suspect that this uncoupling is due to a defect in the signaling pathway downstream of CXCR4. In this context, our results imply that Tregs can affect the activity of PC-intrinsic factors involved in responsiveness to CXCL12. How this happens remains to be clarified.

Another molecular change found in K/BxNsf mice is the downregulation of FcγRIIb expression on splenic PCs. Because FcγRIIb is known to transduce a death signal to PCs upon engagement with immune complexes (29), this downregulation presumably permits them to escape from immune complex-mediated cell death. Indeed, in agreement with this idea, we found that K/BxNsf PCs were resistant to immune complex-mediated cell death. This characteristic was also observed when PCs from FcγRIIb-deficient mice were incubated with FcγRIIb-specific cross-linking Abs (29), so confirming that the escape of K/BxNsf PCs from immune complex-mediated death arises from...
their insufficient expression of FcγRIIB. This escape cannot be attributed to a direct effect of Tregs, as we failed to detect upregulation of FcγRIIB on PCs when Tregs were cocultured with PCs in vitro. Hence, it is likely that factors extrinsic to, and influenced by, Tregs affect FcγRIIB expression on PCs. We suspect that IL-4 may be involved in this phenomenon, because IL-4 has been known to reduce FcγRIIB expression on B cells (39), and K/BxN mice contain more numerous IL-4-producing Th2 cells than do K/BxN mice in our observation (data not shown).

Several studies have identified PCs with aberrant phenotypes in models of SLE, and some of these phenotypes were evident among the PCs from the arthritic K/BxN mice. For instance, autobody-secrating long-lived PCs are abundant in the spleens of NZB/W mice (9). Similarly, the majority of splenic PCs in the NZM2410 mouse strain, another SLE model, are long-lived and defective in BM homing (23). Furthermore, there is a report showing the absence of FcγRIIB expression on PCs from murine SLE models (29). Our data prompted us to hypothesize that the aberrant phenotypes of PCs in SLE are due to a numerical and/or functional deficit in Tregs. Given that a deficit in Tregs has been reported in patients with SLE (40, 41), this hypothesis seems to be worthy of further investigation.

In conclusion, our study highlights the importance of Tregs in the regulation of humoral immunity. To our knowledge, this is the first report showing that Tregs regulate the behavior of PCs as well as the quantity of TEFH cells, TFH cells, and GCs in vivo. From the current observations, we conclude that the activity of Tregs is crucial for regulating PC homeostasis in autoimmune states, thereby dampening humoral memory.

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Disclosures
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