The Journal of Immunology

This information is current as of July 25, 2017.

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

Eunkyeong Jang, Wang Sik Cho, Mi-La Cho, Hyun-Joo Park, Hye-Joa Oh, Sang Mee Kang, Doo-Jin Paik and Jeehee Youn

*J Immunol* 2011; 186:1546-1553; Prepublished online 5 January 2011;
doi: 10.4049/jimmunol.1002942
http://www.jimmunol.org/content/186/3/1546

References
This article cites 41 articles, 21 of which you can access for free at:
http://www.jimmunol.org/content/186/3/1546.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Foxp3+ Regulatory T Cells Control Humoral Autoimmunity by Suppressing the Development of Long-Lived Plasma Cells

Eunkyeong Jang,* Wang Sik Cho, † Mi-La Cho, ‡ Hyun-Joo Park,* Hye-Joa Oh, ‡ Sang Mee Kang,* Doo-Jin Paik, † and Jeehee Youn*†

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/BxNsfs mice. The disease symptoms of K/BxNsfs mice were exacerbated, and this coincided with increases in extrafollicular Th cells, follicular Th cells, and germinal centers. Surprisingly, the K/BxNsfs 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 FcγRIIB 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 FcγRIIB 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: 1546–1553.

Systemic 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 plasmablasts or to the activity of long-lived plasma cells (PCs). 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 germinal centers (GCs) formed in peripheral lymphoid organs, such as spleen and lymph nodes (LNs) (4, 5). The early PC precursors that emerge from these reactions are dividing, rapidly turning over, Ab-secreting plasmablasts, which in turn differentiate into nondividing 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...
disease model named K/BxN. The spontaneous development of severe arthritis in this model depends on the production of auto-
antibodies to glucose-6-phosphate isomerase (GPI) (20). Pre-
viously, we found that despite the loss of self-tolerance, func-
tionally 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, mat-
uration, and life span—which favor the persistence of humoral
immunity. Thus, our results demonstrating PC regulation by Tregs
provide novel insight into how Tregs limit humoral memory.

Materials and Methods

Mice

A cross between KRN TCR-transgenic mice on a C57BL/6 background
(K/B) and NOD mice generated arthritis 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 sf 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/BxNs
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.

FACS analyses

Single-cell suspensions of spleen, joint-draining lymph node (dLN; a pool
of axillary, inguinal, and popliteal LNs), and BM cells were surface or
intracellularly stained as previously described (22). The following mAbs
and reagents were purchased from BD Biosciences: anti-CD4–FITC, anti–GL-7–
PE, anti-CD4–allophycocyanin, anti-B220–allophycocyanin, biotinylated anti-
FITC, and anti-CD138–PE. The following mAbs and reagents were purchased
from BD Biosciences: anti-CD4–FITC, anti–GL-7–
goat serum, and stained with appropriate combination of mAbs. The following
mAbs were purchased from Bio-rad: anti-CD4–FITC, anti-CD138–PE,
anti-CD5–PE, anti-CD6–PE, anti-CD95–PE, anti-CD95L–PE, anti-CD4–FITC,
anti-CD152–PE, anti-CD5–PE, anti-CD95–PE, anti-CD95L–PE, and stained with
appropriate combination of mAbs.

Fluorescence microscopy

Mouse spleens were embedded in OCT compound and snap-frozen in liquid
nitrogen. Cryosections (7 μm thick) were fixed with acetone, blocked with 10% 
goat serum, and stained with appropriate combination of mAbs. The following
mAbs were purchased from BD Biosciences: anti-CD4–FITC, anti-GL-7–
FITC, and anti-CD138–PE. The following mAbs and reagents were purchased
from eBioscience: anti-CD4–PerCP, anti–PSGL-1, anti-CD45RO, FITC–anti–
IFN-γ, PE–anti-CD138, anti-GL-7–PE, and streptavidin–Cy3. Fluorescence images
were acquired using an LSM 510 confocal microscope (Zeiss).

ELISPOT assays

GPI-specific IgG1-secreting cells in spleen and BM were enumerated by
ELISPOT assays as described (22). Aliquots of serial dilutions of cell
susensions from spleens and BM were added to GIP-coated plates and
incubated overnight at 37°C, followed by sequential incubation with bio-
tinylated anti-mouse IgG1 mAb (BD Bioscience) and streptavidin–perox-
idasre. After adding AEC substrate (BD Bioscience), numbers of spots of
GPI-specific IgG1 were enumerated by Fluorescence microscopy.

Cell migration assays

PC chemotaxis was quantitated by ex vivo cell migration assays as described
(23). Splenocytes (3 × 10^5) were added to the inserts of 5-μm microporous
Transwell plates (Corning) and incubated in RPMI 1640 medium con-
taining 10% FBS for 1 h at 37°C. After adding 100 ng/ml mouse CXCL12
(R&D Systems) to the wells, the cells were cultured for an additional 3 h.
Cells remaining in the inserts and those that had migrated to the wells
were collected and assayed by FACS.

RT-PCR

B220+ and B220+CD138+ cells were sorted by MACS (Miltenyi Biotec).
The purity of resulting cell fractions was more than 97%. Total RNA was
purified using TRIZol reagent (Life Technologies). The level of FcyRIIb
mRNA was measured by quantitative RT-PCR using an iCycler thermo-
cycler (Bio-Rad). Relative amounts of FcyRIIb transcripts were normal-
ized to the amounts of β2-microglobulin transcript. The primer sequences
used were as follows: FcyRIIb forward, 5'-GGA AGA CAC GAT GAC
ACT GA-3'; FcyRIIb reverse, 5'-TGC TCC ATT TGA CAC CGA TA-3';
β2-microglobulin forward, 5'-TGA CCA GCT TGT ATG CTA TC-3'; β2-
microglobulin reverse, 5'-CAG TGT GAG CCA GGA TAT AG-3'.

BrDU incorporation assays

Mice were fed drinking water containing 0.8 mg/ml BrdU (Sigma-Aldrich)
for 14–28 d. The water was protected from light and changed every 2 d.
After staining for B220 and CD138, splenocytes and dLN cells extracted from
the mice were fixed, permeabilized, treated with 50 Kunitz units
DNase (Sigma-Aldrich), and then stained with FITC-conjugated anti-BrdU
mAb according to the manufacturer’s instructions (BD Biosciences). BrdU
incorporation in B220+CD138+ cells was determined by FACS analysis.

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
Biosciences) 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 deter-
mine whether Tregs affect FcyRII 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, GPI immune complexes were made by adding 10 μg/
ml GPI 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/BxNs 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 autoimmu-
nity, we generated Treg-deficient K/BxNs mice by congenically
combining the K/BxN mice with Foxp3 mutant scurfy mice. We
found that K/BxNs mice exhibited earlier onset and more ag-
gressive 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/BxNs 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 extra-
ofollicular or follicular areas. These two independent responses
are governed by two distinct populations of Th cells, CD4+CD44hi
CD62LloPSGL-1lo TEFH cells and CD4+CD25+CXCR5+ICOS+ TFH
(4, 5). Both populations emerged in the spleen and dLNs of
A cross between KRN TCR-transgenic mice on a C57BL/6 background
(K/B) and NOD mice generated arthritis 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 sf 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/BxNs
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.
FIGURE 1. Enhanced development of $T_{EFP}$ and $T_{FH}$ cells and GCs in K/BxNsF mice. A, Splenocytes (Sp) and dLN cells from BxN, K/BxN, and K/BxNsF mice were stained for CD4, CD44, CD62L, and PSGL-1. FACS profiles gated on CD4+CD138+ cells are shown. A representative result for 8-wk-old mice for the percentage of CD62LloPSGL-1lo cells among CD4+CD138+ cells is shown (top panels). Graphs are means ± SEM of CD4+CD134+CD44hiCD62LloPSGL-1lo cell numbers in spleen and dLNs (bottom panels). *p < 0.05; **p < 0.01 (Student t test). C, Immunofluorescence staining of spleen cryosections with anti–CD4–FITC (green), anti–B220–allophycocyanin (blue), and anti–GL-7–Cy3 (red). D, Immunofluorescence staining of spleen cryosections with anti–CD4–Cy3 (red), anti–B220–allophycocyanin (blue), and anti–GL-7–FITC (green). E, Immunofluorescence staining of spleen cryosections with anti–CD4–allophycocyanin (blue), anti–GL-7–FITC (green), and anti–Foxp3–PE (red). Foxp3+ cells in the GC are indicated by the arrows.

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 predominately of CD138+CD134hi early PCs (plasmablasts) rather than CD138+CD134lo mature PCs (Fig. 2A, 2B). Second, a substantial fraction of autotaxin-secretion-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 expressing its receptor normally (B220lo) PCs from K/BxNsF mice expressed even higher CD44hiCD62LloPSGL-1lo cell numbers in CD4+CD25+ cells is shown (top panels). Graphs are means ± SEM of CD4+CD25hi cells from K/BxN, K/BxNsF and K/BxNsf mice stained for CD4, CD44, CD62L, and PSGL-1. FACS profiles gated on CD4+CD25hi cells are shown. A representative result for 8-wk-old mice for the percentage of CXCR5+ICOS+ cells among CD4+CD25+ cells is shown (top panels). Graphs are means ± SEM of CD4+CD25hi CXCR5+ICOS+ cell numbers in spleen and dLNs (bottom panels). *p < 0.05; **p < 0.01 (Student t test). C, Immunofluorescence staining of spleen cryosections with anti–CD4–FITC (green), anti–B220–allophycocyanin (blue), and anti–GL-7–Cy3 (red). D, Immunofluorescence staining of spleen cryosections with anti–CD4–Cy3 (red), anti–B220–allophycocyanin (blue), and anti–GL-7–FITC (green). E, Immunofluorescence staining of spleen cryosections with anti–CD4–allophycocyanin (blue), anti–GL-7–FITC (green), and anti–Foxp3–PE (red). Foxp3+ cells in the GC are indicated by the arrows.

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 PCs did (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 (B220lo) and late (B220hi) 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 are 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 FcγRIIb-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 FcRIIb expression affected immunecomplex-mediated cell death of the K/BxNsf PCs, purified splenic PCs from K/BxNsf mice were subjected to ex vivo migration assays. Cells loaded in the upper Transwells (input) were inserted into lower wells containing medium or 100 ng/ml CXCL12. After 3 h, the cells in the lower wells were collected and assayed by FACS. B, Spleen cells from 5- to 8-wk-old BxN, K/BxN, and K/BxNsf mice contain stained with mAbs to B220, CD138, CXCR4, and VLA4. FACS profiles gated on either B220+CD138+ plasmablasts or B220+CD138+ PCs are shown. The numbers indicate the percentage of B220+CD138+ cells among viable lymphocytes. One representative result of three independent experiments is shown.

To answer this question, we first measured the level of FcRIIb expression on the PCs and found that the surface level of FcRIIb 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+ cells as a whole and in B220+CD138+ 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/BxNsf mice were incubated with a mixture of GPI and normal 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+/7-AAD−) 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/K/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+CD138+ 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 Tfh and Th17 cells in K/BxNsf mice imply that Tregs in regulating the quantity of these cells in vivo. The abundance of Th17 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...
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, the functions of TFH cells are attenuated by the induction of Tregs, Foxp3+ Tregs (35, 36). Moreover, although FOXP3+ Tregs isolated from human tonsil limit certain functions of TFH cells in vivo. Hence, our results provide new insight into the in vivo role of Foxp3+ Tregs in contact with CD138+ PCs in the extrafollicular zone of spleen sections with anti-CD4–PerCP–Cy5.5 (white), anti-Foxp3–FITC (green), anti-B220–allophycocyanin (blue), and anti-CD138–PE (red). Foxp3+ Tregs in contact with CD138+ PCs in the extracellular zone of spleen are indicated by the arrows. B and C, 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).

sensitivity 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 CXCL12-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 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

FIGURE 5. Cytotoxic effect of Tregs on PCs is elicited in a contact-dependent manner. A, Immunofluorescence staining of K/BxN spleen cryosections with anti-CD4–PerCP–Cy5.5 (white), anti-Foxp3–FITC (green), anti-B220–allophycocyanin (blue), and anti-CID138–PE (red). Foxp3+ Tregs in contact with CD138+ PCs in the extracellular zone of spleen are indicated by the arrows. B and C, 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).
their insufficiency 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/BxNs 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/BxNs mice. For instance, autoantibody-secreting 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 Tfh 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.

Acknowledgments
We thank Dr. Diane Mathis for providing KN mice, Dr. Hee Yong Chung for valuable discussion, and Dr. Julian Gross for editorial assistance.

Disclosures
The authors have no financial conflicts of interest.

References

