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CUTTING EDGE

Cutting Edge: Low-Affinity, Smith Antigen-Specific B Cells Are Tolerized by Dendritic Cells and Macrophages[1]

Michelle A. Kilmon, Jennifer A. Rutan, Stephen H. Clarke, and Barbara J. Vilen[2]

Polyclonal B cell activation promotes immunity without the loss of tolerance. Our data show that during activation of the innate immune system, B cell tolerance to Smith Ag Sm is maintained by dendritic cells (DCs) and macrophages (MΦ). TLR4-activated myeloid DCs and MΦ, but not plasmacytoid or lymphoid DCs, repressed autoreactive B cells through the secretion of soluble mediators, including IL-6. Although IL-6 promotes plasma cell differentiation of B cells acutely stimulated by Ag, we show that it repressed cells that were chronically exposed to self-Ag. This mechanism of tolerance was not limited to Smith Ag-specific B cells as hen egg lysozyme- and p-azophenylarsonate-specific B cells were similarly affected. Our data define a tolerogenic role for MΦ and DCs in regulating autoreactive B cells during activation of the innate immune system. ***The Journal of Immunology,*** **2005, 175: 37–41.**

Toll-like receptors regulate innate host defense and control adaptive immune responses. These receptors detect microorganisms by recognizing pathogen-associated molecular patterns such as hypomethylated DNA containing CpG motifs, viral nucleic acids, and LPS. During innate immune responses, polyclonal B cell activation facilitates rapid and efficient Ab secretion to eliminate invading pathogens. Despite polyclonal B cell activation, most individuals do not succumb to autoimmunity, suggesting that tolerance mechanisms regulate autoreactive B cells during innate immune responses. In support of this, a recent report showed that TLR9-mediated responses were regulated by continuous BCR-mediated ERK activation, with uncoupling of the BCR from a calcineurin-dependent pathway. This provided the first evidence that self-Ags regulated autoreactive B cells during activation of the innate immune system (1).

Dendritic cells (DCs)[3] and macrophages (MΦ) regulate the balance between immunity and tolerance by controlling lymphocyte activation. In the absence of pathogens, DCs maintain T cell unresponsiveness by presenting self-Ags without costimulation (2). In addition, MΦ suppress T cell activity (3). In contrast, pathogen ligation of TLRs activates DCs and MΦ by inducing costimulatory molecules and promoting cytokine secretion. A recent report (4) described that activation of the innate immune system induced DCs and MΦ to secrete IL-6, making helper T cells refractory to the suppressive effects of regulatory T cells. Thus, TLR-activated DCs and MΦ promote immunity by inducing polyclonal activation of the T cell repertoire.

DCs and MΦ also regulate B cell immunity. Cytokines secreted by plasmacytoid DCs (pDCs) induce activation and promote differentiation of activated B cells into Ab secreting cells (5). In addition, DCs engulf particulate bacteria and secrete B lymphocyte stimulator/April to activate marginal zone (MZ) B cells and B1 cells (6). Similarly, B lymphocyte stimulator, secreted by MΦ, enhances BCR-mediated proliferation (7). Despite emerging evidence that DCs and MΦ play a role in B cell immunity, their significance in regulating B cell tolerance has not been reported.

In the present study, we describe a novel mechanism of B cell tolerance that is reversible and mediated by myeloid DCs (myDCs) and MΦ. We found that DCs secreted IL-6, which repressed Ig secretion by B cells chronically exposed to self-Ag but not by naive B cells. The repressive ability of IL-6 was not limited to Smith Ag (Sm)-specific B cells because chronically activated hen egg lysozyme (HEL)-specific B cells and *p*-azophenylarsonate (Ars)-specific B cells were repressed. Similar to DCs, MΦ secreted a soluble factor that repressed autoantibody secretion. This factor was not neutralized by anti-IL-6, indicating that another soluble mediator(s) repressed Ig secretion. In summary, our findings define a tolerogenic role for DCs and MΦ in repressing autoantibody secretion and reveal a role for chronic Ag exposure in reprogramming IL-6 responsiveness.

Materials and Methods

Mice

2-12H/Vκ8/C$\kappa^{-/-}$ mice were generated by mating 2-12H/C$\kappa^{-/-}$ mice (C57BL/6 × C.B17) and Vκ8/C$\kappa^{-/-}$ mice (C57BL/6) (8). Ars/A1 mice

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[3] Abbreviations used in this paper: DC, dendritic cell; myDC, myeloid DC; pDC, plasmacytoid DC; MZ, marginal zone; MΦ, macrophage; non-Tg, nontransgenic; CM, conditioned medium; BMDC, bone marrow-derived DC; Sm, Smith Ag; HEL, hen egg lysozyme; BMMΦ, bone marrow-derived MΦ; lyDC, lymphoid DC; Ars, *p*-azophenylarsonate; SLE, systemic lupus erythematosus.

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(C57BL/6 × A/J) (9) were provided by Dr. L. Wysocki (National Jewish Medical Center, Denver, CO.). HEL-Ig and HEL-Ig × sHEL mice (C57BL/6) (10) and C57BL/6 (nontransgenic (non-Tg)) mice were purchased from The Jackson Laboratory. Animals were 8–16 wk old and maintained in an accredited animal facility.

Reagents and Abs

Antibodies to CD11c, CD11b, B220, CD3, NK1.1, IL-6, and rIL-6 were purchased from BD Biosciences; antibodies to GR1, DX5, and streptavidin were purchased from eBioscience; anti-IgM^b was obtained from Southern Biotechnology; GM-CSF, IL-4, and M-CSF were purchased from PeproTech, and *Escherichia coli* 055:B5 LPS was obtained from Sigma-Aldrich. B7.6 (anti-IgM) and 54.1 (3-83 Id), an isotype control for anti-IL-6, were purified from hybridoma supernatant.

Cell purification

Splenic B cells were isolated by negative selection (StemCell Technologies). B cells were 90–93% pure as determined by flow cytometry. DCs (anti-CD11c) and MΦ (anti-CD11b) were purified from non-Tg mice by positive selection (Miltenyi Biotec). Both populations were 70% pure with 20% lymphocyte contamination. The MΦ were contaminated with 10% DCs; DCs were contaminated with 10% MΦ. MΦ were I-A^{low} and B7.2^{low}. DCs were I-A^{int} and B7.2^{low}. MΦ and DC subsets were separated based on expression of CD11c, CD11b, B220, and GR1. T, NK, and NK T cells were separated based on expression of CD3, DX5, and NK1.1.

LPS stimulation

Splenocytes (1×10^5 B cells) or equivalent numbers of purified B cells (purity determined by flow cytometry) were cultured with 30 μg/ml LPS. DCs, MΦ, T cells, NK cells, NKT cells, rIL-6, DC and MΦ conditioned medium (CM) (25% of final volume), or anti-IL-6 were added at day 0.

ELISA

IgM^a/κ (encoded by 2-12H/Vκ8 or Ars/A1) and mouse anti-HEL IgM^a were measured as described previously (8, 11). Mouse anti-HEL IgM^a was a gift from Dr. T. Tedder (Duke University, Durham, NC) (11). IgM^b was detected using anti-mouse IgM^b and anti-mouse IgM-biotin. Data were plotted as either total IgM^a/κ levels or as percentage of control calculated as the percent secretion relative to cultures of LPS-stimulated B cells.

Proliferation assay

B cell proliferation was determined by stimulating 1×10^5 purified B cells with LPS in the presence or absence of DCs (5×10^3) or rIL-6 (10 ng). The cultures were pulsed with 1 μCi/well [^{3}H]thymidine (Dupont/NEN) during the last 8 h of a 72-h incubation. Data were plotted as percent of control calculated as the percent proliferation relative to cultures of LPS-stimulated B cells.

Bone marrow-derived DCs (BMDCs) and MΦ (BMMΦ) cultures

Bone marrow single-cell suspensions were prepared from the femurs of non-Tg mice. Following RBC lysis, cells were cultured in GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) for 5 days. BMDC cultures were 99% CD11c$^+$, I-A^{low}, and B7.2^{low}. BMMΦ (98% pure) generated by culturing bone marrow in M-CSF (20 ng/ml) for 1 wk were I-A^{neg} and B7.2^{neg}. CM was made from 1×10^4 BMDCs or BMMΦ cultured for an additional 4 days with or without LPS (30 μg/ml).

Results

B cells from mice expressing the 2-12 Ig H chain transgene (IgM^a) paired with a Vκ8 L chain transgene bind Sm with low affinity and are tolerized by peripheral anergy (8). During the characterization of anergic, Sm-specific B cells, we unexpectedly observed that purified B cells secreted IgM^a/κ in response to LPS, whereas unpurified splenocytes remained LPS unresponsive (Ref. 8; Fig. 1*A*). Interestingly, reconstitution of purified Sm-specific B cells with B cell-depleted splenocytes (henceforth called non-B cells) repressed IgM^a/κ secretion. Non-B cells from non-Tg mice also inhibited secretion, indicating that repression was not specific to 2-12H/Vk8 mice (Fig. 1*A*). These data show that the unresponsive phenotype of Sm-specific B cells is reversible and conferred by non-B cells.

To identify the regulatory cell(s) that repressed Ig secretion, ex vivo DCs, MΦ, T cells, NK cells, and NK T cells were cocultured with purified Sm-specific B cells. T, NK, and NK T cells failed to inhibit LPS-induced IgM^a/κ secretion (Fig. 1*B*). However, ex vivo DCs (CD11c$^+$) and MΦ (CD11b$^+$) repressed 75% of secretion when one DC or MΦ was cocultured with 10 B cells (Fig. 1*B*). To confirm that MΦ and DCs repressed Ig secretion, BMMΦ and BMDCs were cocultured with Sm-specific B cells at increasing B:DC ratios. BMMΦ and BMDCs repressed ~95% of IgM^a/κ secretion when cultured at a ratio of 10:1 (10 B cells to one BMMΦ or BMDC) (Fig. 1*C*). At 100:1, BMMΦ repressed 85% of IgM^a/κ, whereas BMDCs repressed only 38%. These data indicate that DCs and MΦ maintain autoreactive B cells in an unresponsive state but that BMMΦ are more potent repressors than BMDCs.

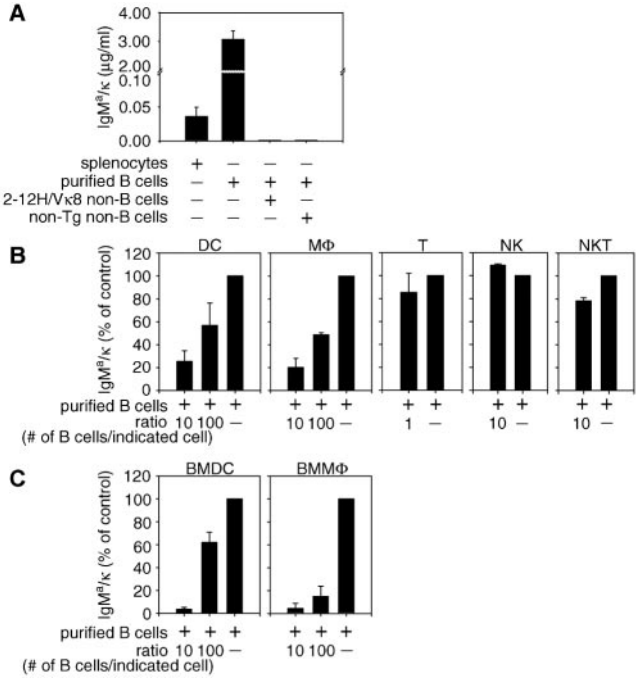

FIGURE 1. DCs and MΦ repress Ig secretion by Sm-specific B cells. Splenocytes from 2-12H/Vκ8 mice (1×10^5 B cells) or equal numbers of purified B cells were stimulated with LPS (30 μg/ml) or cocultured with 1×10^5 non-B cells from either 2-12H/Vκ8 or non-Tg mice and (*A*), the indicated ratio of positively selected splenic DCs and MΦ, or sorted T, NK, or NK T cells (*B*), or BMDCs or BMMΦ from non-Tg mice (*C*). IgM^a/κ levels were quantitated at day 4 by ELISA. LPS-stimulated B cells (100%) secreted 2–10 μg/ml IgM^a/κ. Data represent at least four experiments.

To identify the subsets of CD11c$^+$/CD11b$^+$ cells responsible for DC/MΦ-mediated repression, MΦ, myDCs, lymphoid DCs (lyDCs), and pDCs were purified from non-Tg mice and cocultured with Sm-specific B cells (Fig. 2*A*). As shown in Fig. 2*B*, both lyDCs and pDCs failed to repress Ig secretion. In contrast, myDCs repressed 48% and MΦ repressed 83% of LPS-induced IgM^a/κ secretion when cultured at a ratio of 10:1 (10 B cells to 1 myDC or MΦ). At a ratio of 20:1, splenic myDCs repressed only 29% of secretion, whereas splenic MΦ repressed 70%. These data identify myDCs and MΦ as regulatory cells that repress LPS-induced Ig secretion and corroborate the findings that splenic MΦ and BMMΦ are more efficient at repressing Ig secretion than splenic DCs and BMDCs.

The low numbers of DCs and MΦ required to repress Ig secretion suggested that a soluble factor may be responsible. To test this, we cocultured Sm-specific B cells with CM from unstimulated and LPS-stimulated DCs and MΦ. LPS-stimulated

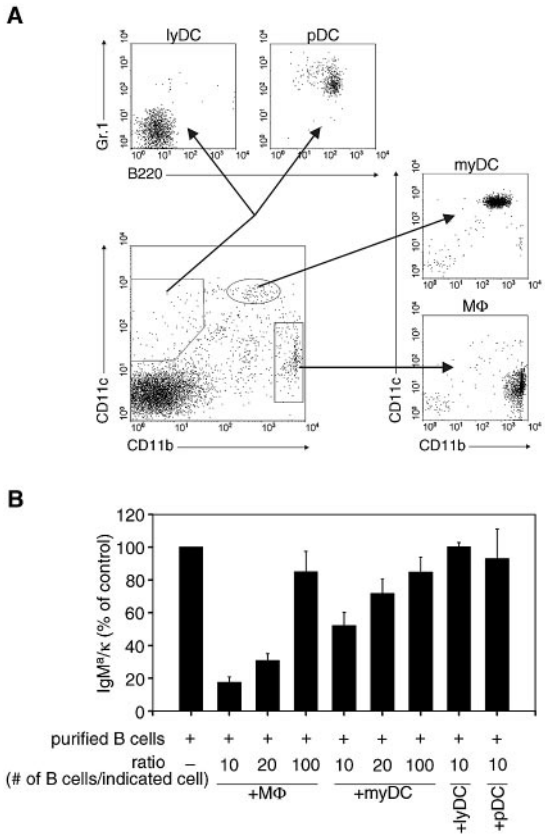

FIGURE 2. Ig secretion by Sm-specific B cells is repressed by myDCs and MΦ but not other DC subsets. *A*, Splenic myDCs, lyDCs, pDCs, and MΦ were sorted based on expression of CD11c, CD11b, B220, and GR1. *B*, Various ratios of myDCs, lyDCs, pDCs, and MΦ were cocultured with Sm-specific B cells (1×10^5) and stimulated with LPS (30 μg/ml). IgM^a/κ secretion was quantitated by ELISA. LPS-stimulated B cells (100%) secreted 2–25 μg/ml IgM^a/κ. Data represent at least three experiments.

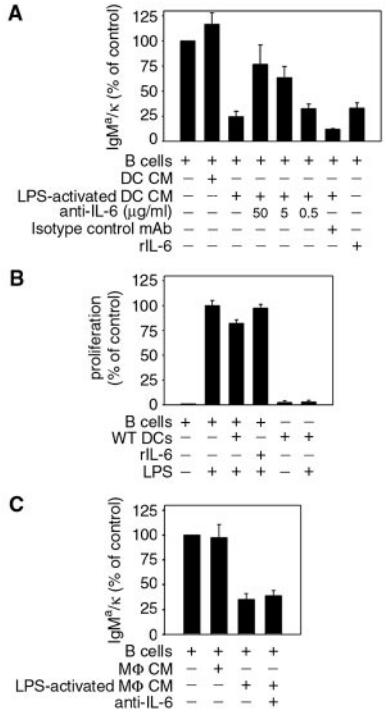

FIGURE 3. A soluble factor represses Ig secretion from Sm-specific B cells. *A*, Sm-specific B cells (1×10^5) were stimulated with LPS (30 μg/ml) in the presence of either DC CM, LPS-activated DC CM, anti-IL-6, isotype-matched control mAb (50 μg/ml), or rIL-6 (10 ng/ml). IgM^a/κ secretion was quantitated by ELISA. *B*, Unstimulated or LPS-stimulated Sm-specific B cells (1×10^5) were cultured with DCs (5×10^3) or rIL-6 (10 ng/ml). Proliferation was determined on day 3 by $[^3H]$thymidine incorporation. The data from four experiments were plotted as percent proliferation relative to LPS-stimulated B cells (counts per minute range = 75,000–130,000). *C*, Sm-specific B cells were stimulated with LPS in the presence of MΦ CM, LPS-activated MΦ-CM, or anti-IL-6 (50 μg/ml). IgM^a/κ was quantitated by ELISA. LPS-stimulated B cells (100%) secreted 2–25 μg/ml IgM^a/κ. Data represent at least three experiments.

DC CM, but not unstimulated DC CM, inhibited 75% of IgM^a/κ secretion (Fig. 3*A*). Neutralization of IL-6 restored IgM^a/κ to 75% of control while an unrelated Ab failed to relieve repression (Fig. 3*A*). Furthermore, rIL-6 repressed 67% of IgM^a/κ secretion, confirming that IL-6 repressed Ig secretion. Repression by DCs and rIL-6 was not due to decreased proliferation because B cells cocultured with or without DCs or IL-6 showed comparable proliferation (Fig. 3*B*). Interestingly, LPS-stimulated MΦ CM was not neutralized by anti-IL-6 (Fig. 3*C*), despite the presence of IL-6 (data not shown). This indicates that in addition to IL-6, MΦ secrete a second factor that represses Ig secretion. This may explain the findings that MΦ are more efficient at repressing Ig secretion than DCs (Figs. 1*C* and 2*B*).

We were puzzled by our findings that IL-6 repressed Ig secretion by Sm-specific B cells when previous reports demonstrated that IL-6 promoted plasma cell differentiation (12). We hypothesized that chronic Ag exposure might alter the response of B cells to IL-6. To test this, we compared the effects of IL-6 on naive B cells and chronically Ag-experienced B cells. As shown in Fig. 4*A*, B cells from non-Tg mice secreted Ig in response to LPS and were minimally affected by coculture with CM from LPS-stimulated DCs or rIL-6. Similarly, LPS-induced Ig secretion by naive HEL-specific B cells (HEL-Ig) was similar in the presence or absence of IL-6 (Fig. 4*B*). However, chronically Ag-experienced B cells from HEL-Ig × sHEL mice showed a 57% reduction in secreted Ig under the same conditions (Fig. 4*C*). These data show that continuous exposure of autoreactive B cells to self-Ag induces susceptibility to IL-6-mediated repression.

B cells from Ars/A1 IgTg mice bind Ars and ssDNA (9). To assess if DC-mediated repression affects B cells of other self-reactive specificities, we tested whether these cells were repressed by IL-6. Unpurified splenocytes from Ars/A1 mice failed to secrete Ig upon LPS stimulation, consistent with an anergic state. However, purified Ars-specific B cells reversed their unresponsive state permitting IgM^a/κ secretion (Fig. 4*D*). Addition of rIL-6 or CM from LPS-stimulated DCs repressed 74% of Ig secretion. Although Ig secretion by B cells from Ars/A1 mice was lower (average = 3.8 μg/ml) than B cells from non-Tg (average = 29.6) and HEL-Ig (average = 20.6), the levels were similar to Ig secretion by Sm-specific B cells (average = 4.6 μg/ml). These results show that Ig secretion by chronically Ag-experienced B cells is regulated by cytokines, representing the first evidence that IL-6 represses Ig secretion by autoreactive B cells.

Discussion

Our data demonstrate that DCs and MΦ play an important role in maintaining tolerance of autoreactive B cells. Previous

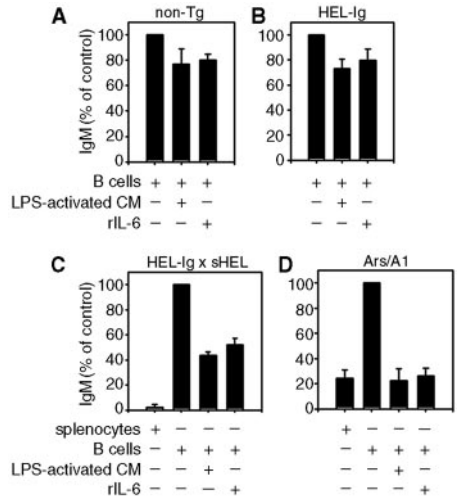

FIGURE 4. Chronically Ag-experienced but not naive B cells are susceptible to repression by IL-6. Splenocytes or purified B cells (1×10^5) from non-Tg (*A*), HEL-Ig (*B*), HEL-Ig x sHEL (*C*), or Ars/A1 mice (*D*) were stimulated with LPS (30 μg/ml) alone or in the presence of LPS-activated DCCM or rIL-6 (10 ng/ml) for 4 days. IgM^b (non-Tg), anti-HEL IgM^a, and IgM^a/κ (Ars/A1) were quantitated by ELISA. From four experiments, LPS-stimulated B cells (100%) secreted 14–42 μg/ml Ig (*A*), 7–27 μg/ml (*B*), 8–32 μg/ml (*C*), and 2–5 μg/ml (*D*).

studies showed that low-affinity, Sm-specific B cells remained unresponsive to TLR4 ligation (8). Our data extend these observations to show that repression of Ig secretion was mediated by DCs and MΦ. DCs and MΦ mediated their effects through the secretion of multiple soluble factors, including IL-6. Interestingly, only B cells chronically exposed to self-Ag were susceptible to IL-6-mediated repression. These data underscore the importance of DCs and MΦ in maintaining the balance between immunity and tolerance and reveal that IL-6 has pleiotropic effects on B cells. A recent report (4) showed that IL-6, secreted by activated DCs and MΦ, activated $CD4^+$ Th cells by blocking the suppressive effects of T regulatory cells. Our data show that IL-6 represses Ig secretion in B cells. Collectively, a model emerges wherein bacterial or viral infections induce DCs and MΦ to secrete IL-6. This leads to polyclonal activation of naive B and T cells (4, 12). Simultaneously, IL-6 acts on autoreactive B cells to suppress Ig secretion, thereby promoting tolerance and immunity during polyclonal activation.

Although two models of tolerance to Sm have been described previously, this study focuses on the low-affinity, Sm-specific B cells from 2-12H/Vκ8 mice (8). The 2-12H and Vκ8 L chains were identified from Sm-specific hybridomas derived from MRL/*lpr* mice. The splenic B cell populations from 2-12H/Vκ8 mice are comprised of 80% follicular, 17% transitional, and 1% MZ B cells. In the 2-12H model, the H chain transgene pairs with endogenous L chains, resulting in receptor/Ag interactions of varying affinities. We detect binding of recombinant Sm to 15–35% of B cells, suggesting they express higher affinity receptors. The percentage of cells that bind Sm with low affinity is unknown because this binding is undetectable by flow cytometry. Nonetheless, these cells exist because sequencing of Sm hybridomas from 2-12H mice indicates the presence of Vκ8 L chains. The spleens of 2-12H mice are comprised of 60% follicular, 10% transitional 1, and 17% MZ B cells (13). Unlike B cells from 2-12H/Vκ8 mice, some B cells in 2-12H mice remain functional because immunization with small nuclear ribonucleoproteins or stimulation with LPS induced the secretion of anti-Sm Abs, albeit at a reduced level compared with non-Tg mice (14). Ig secretion may be derived from transitional B cells (14) or possibly MZ cells (unpublished observations). Despite some functional B cells, other B cells from 2-12H mice are regulated by repression because purified, LPS-stimulated B cells from 2-12H mice secreted more Ig compared with nonpurified splenocytes (unpublished observations). Collectively, the data suggest that DC/MΦ-mediated repression regulates Ig secretion by low-affinity B cells within the 2-12H model; however it remains unclear if higher affinity B cells use this tolerance mechanism.

Receptor cross-talk is emerging as an important mechanism for modulating signal transduction. Our data show that IL-6 repressed Ig secretion in B cells chronically exposed to Ag, suggesting that the BCR modulates IL-6R signaling. In support of this, signal transduction through the epidermal growth factor and IFN-$\alpha\beta$Rs modulates IL-6R signaling (15, 16). In addition, BCR-mediated ERK activation regulates TLR9-induced Ig secretion (1). These findings support the notion that chronic BCR signaling reprograms IL-6 responses, leading to repression of Ig secretion. However, autoreactive B cells that overcome tolerance are activated by IL-6 to terminally differentiate into plasma cells. This is consistent with previous work showing that IL-6 induces anti-DNA Ab secretion by B cells derived from old $(NZB \times NZW)F_1$ but not from young mice. This correlates directly with the age when mice show signs of disease (17). Thus, IL-6 represses Ig secretion in B cells chronically exposed to Ag but induces Ig secretion by cells that have overcome tolerance.

Elevated serum IL-6 levels have been found in many Systemic Lupus Erythematosus (SLE) patients and have been suggested to play a role in the pathogenesis of the disease. In contrast, our data describe a tolerogenic role for IL-6. It remains unclear why systemic IL-6 is not repressive. One explanation is that localized release between regulatory DC/MΦ and B cells may be necessary to achieve sufficient concentrations to repress Ig secretion. Our data show that 10 ng/ml rIL-6 is required to repress 70% of Ig secretion (Fig. 3*A*). Clinical reports indicate that serum IL-6 levels in SLE patients average 9 pg/ml compared with 4 pg/ml in healthy controls (18). Thus, although the elevated IL-6 levels in SLE patients may be sufficient to cause inflammation, the serum concentrations are significantly lower than is required to repress Ig secretion in vitro. We propose that in hosts without a genetic predisposition to disease, activation of the innate immune system induces IL-6 secretion by DCs repressing colocalized, autoreactive B cells. In animals predisposed to disease, tolerance is reversed, possibly due to insufficient release of soluble mediators or insufficient numbers of DC or MΦ. However, once tolerance is overcome, autoantibody secretion promotes immune complex formation, which induces chronic production of proinflammatory mediators, including IL-6, by endothelial cells (19). In support of this model, we have found that DCs from lupus-prone mice are defective in repressing Ig secretion, coincident with defects in IL-6 secretion by DCs. (M. Gilbert, D. Carnathan, and B. J. Vilen, manuscript in preparation). Thus, IL-6 has diverse effects; it promotes inflammation, potentially exacerbating disease, yet balances immunity and tolerance by differentially regulating plasma cell differentiation.

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Disclosures

M. Kilmon, J. Rutan, and B. J. Vilen are among the inventors of pending patent application #WO 2005/027841, entitled "Cells, Compositions and Methods for Repressing B cell Autoantibody Secretion and for Treating Autoimmune Disease."

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