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*J Immunol* 2006; 177:5337-5346;
doi: 10.4049/jimmunol.177.8.5337
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ERK Signaling Is a Molecular Switch Integrating Opposing Inputs from B Cell Receptor and T Cell Cytokines to Control TLR4-Driven Plasma Cell Differentiation

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Differential of B cells into plasma cells represents a critical immunoregulatory checkpoint where neutralizing Abs against infectious agents must be selected whereas self-reactive Abs are suppressed. Bacterial LPS is a uniquely potent bacterial immunogen that can bypass self-tolerance within the T cell repertoire. We show here that during LPS-induced plasma cell differentiation, the ERK intracellular signaling pathway serves as a pivotal switch integrating opposing inputs from Ag via BCR and from the two best characterized B cell differentiation factors made by T cells, IL-2 and IL-5. Continuous Ag receptor signaling through the RAS/MEK/ERK pathway, as occurs in self-reactive B cells, inhibits LPS induction of Blimp-1 and the plasma cell differentiation program. Differentiation resumes after a transient pulse of Ag-ERK signaling, or upon inactivation of ERK by IL-2 and IL-5 through induction of dual-specificity phosphatase 5 (Dusp5). The architecture of this molecular switch provides a framework for understanding the specificity of antibacterial Ab responses and resistance to bacterially induced autoimmune diseases such as Guillain-Barré syndrome. 

The Journal of Immunology, 2006, 177: 5337–5346.
known how the pathway interconnects with the opposing effects of plasma cell differentiation-promoting cytokines from T cells, of which IL-2 and IL-5 are the best characterized. Because ERK signaling is also induced by binding of foreign Ags and by many other receptors, the upstream elements and timing of BCR-ERK signaling responsible for inhibiting plasma cell differentiation remain to be defined. Here, we address these issues by finding that the ERK pathway is a central switch in LPS-induced Blimp-1 expression and plasma cell differentiation. BCR signaling through RAS and MEK to ERK acts as the integration point for two key variables that help to distinguish between binding of self Ags and binding to Ags of acute bacterial pathogens: the kinetics of Ag exposure; and the presence of differentiation cytokines from T cells.

Materials and Methods

Mice and lymphocyte purification

Splenic B lymphocytes were from transgenic mice carrying the MD4 anti-hen egg lysozyme (HEL) Ig H+L transgenes (naive B cells) or together with the ML5-soluble HEL transgene (HEL-anergic B cells) and purified by depletion of non-B cells as described (39). The dominant negative N17™ transgenic mice were previously described (40) and were the gift of Drs. R. Perlmutter (Amgen) and B. Iritani (University of Washington, Seattle, WA). All experiments were performed under approved protocols of the Animal Ethics and Experimentation Committee of Australian National University.

Western blot analysis

Analysis of ERK, Blimp-1 was performed as described (41). Briefly, the purified splenic B cells at a density of 2 x 10⁶/ml were stimulated at 37°C. After stimulation, cells were washed with ice-cold PBS and then lysed on ice in the lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 5 mM Na₂EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM EGTA, 10 mM sodium fluoride, 1 mM aprotinin, 1 mM leupeptin, 1 mM Na₃VO₄, 1 mM tetrathiophosphate, and 100 µM 3'-γ-γ-glycerophosphate. The lysates were centrifuged at 13,000 rpm, and the supernatants were removed and boiled in 5× SDS sample buffer (10% SDS, 50% glycerol, 0.2 M Tris-HCl (pH 6.8), 5% 2-ME, and bromphenol blue to color). Protein samples were separated on SDS-PAGE, transferred to nitrocellulose membrane, and then blotted with anti-phospho-ERK Abs (Cell Signaling Technology) following the manufacturer’s protocol or antisera to Blimp-1. Blots were reprobed with anti-ERK Abs (Cell Signaling Technology) to ensure that all reactions contained equal amounts of ERK.

Retroviral transduction of B cells and culture conditions

A cDNA encoding mutant gain-of-function MEK* (42), containing two point mutations (G12R, A12S) and a deletion of residues 32–52 of the N-terminal region, was subcloned into the polylinker of the Moloney leukemia virus long terminal repeat retroviral vector. The constructs were transfected into Phoenix cells (a gift from G. Nolan, Stanford University, Stanford, CA) by the standard calcium phosphate method. After transfection for 2 days, the supernatant containing viral particles was collected in DMEM supplemented with 10% heat-inactivated FCS, 100 U of penicillin, and 100 µg/ml streptomycin (all from Invitrogen Life Technologies). The supernatants were then filtered through a 0.45-µm pore filter, and the viral supernatant containing 4 × 10⁶ transducing units/ml was added to cultures at 37°C in a 5% CO₂-humidified incubator and maintained in RPMI 1640 (JRH Bioscience) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 10 mM HEPES (pH 7.4), 50 µM 2-ME, 100 U of penicillin and 100 µg/ml streptomycin (all from Invitrogen Life Technologies). For retroviral transduction, splenic B cells were cultured as above with 20 µg/ml LPS (Fluka). For cultures exposed to HEL, HEL was added during these initial periods and during spin occlusion. After 16 h, the cells were spin occluded with the viral supernatant containing 4 µg/ml polybrene (Sigma-Aldrich) and 20 µg/ml LPS. After 12 h, the spin occlusion procedure was repeated; 12 h after the last spin occlusion, the cells were cultured in fresh medium containing 20 µg/ml LPS or in combination with 500 ng/ml HEL. MEK inhibitor PD98059 (Cell Signaling Techn. Inc) was added to culture medium at a final concentration of 20 µM in all experiments.

Flow cytometry

After 4 or 5 days of culture, the cells were stained with Abs in FACS buffer (PBS, 2% FCS, 0.1% sodium azide) to one or more of the following markers: B220, CD72, syndecan-1, and IgM* (all obtained from BD Pharmingen). For selectively staining intracellular IgM*, the cells were first incubated with unlabeled anti-IgM* at a concentration established to saturate all surface Ig binding sites, then fixed in PBS containing 2% paraformaldehyde, followed by incubating with PE-labeled anti-IgM* (BD Pharmingen) in FACS buffer containing 0.5% saponin (Sigma-Aldrich). 7-Aminoactinomycin D (Molecular Probes) was used to determine cell viability.

Proliferation assay

Cells were plated in triplicate at 2 × 10⁶/well in 96-well round-bottom plates in the presence of the indicated concentration of LPS for 60 h. The varying concentrations of cyclosporin A (Novartis) were added along with 0.5 µg/ml or 20 µg/ml LPS in the presence or absence of 500 ng/ml HEL. Cells were pulsed with 1 µCi/well [³H]TdR (ICN) for the last 16 h, harvested onto glass-fiber filters (Packard Bioscience), and the amount of incorporated [³H]TdR was determined using a topcount reader (Packard Bioscience).

ELISA and ELISPOT assay

Anti-HEL IgM Abs were measured as previously described (21). Briefly, 96-well flat-bottom plates were coated with HEL (10 µg/ml) in 0.05 M bicarbonate buffer (pH 9.6) overnight. Plates were blocked with 1% BSA in PBS for 1.5 h at 37°C. Supernatants from day 5 culture diluted in 0.1% BSA-PBS were added to the plates and incubated at 37°C for 1 h. Alkaline phosphatase-conjugated goat anti-mouse IgM Ab was diluted in 0.1% BSA-PBS, and 100 µl were added per well. Plates were incubated at 37°C for 1 h. To develop, nitrophenyl phosphate (5-mg tablets; Sigma) was prepared at 1 mg/ml in nitrophenyl phosphate buffer, and 100 µl were added to each well. After visible color change, optical density was measured at 405 nm using a 96-well plate reader (THERMOMax Molecular Devices). ELISPOT assays for Ab-forming cells were performed as described (12). Briefly, 1 mg/ml HEL in 0.05 M bicarbonate buffer (pH 9.6) was bound to 24-well tissue culture plates. After blocking with 1% BSA in PBS, HEL-cell suspensions were added and cultured for 4–7 days at 37°C in a 5% CO₂ incubator. After washing with PBS supplemented with 0.05% Tween 20, the plates were blocked with a mixture of BSA and skim milk powder at 37°C for 30 min. First biotinylated anti-IgM* (clone RS3.1) was added at 4°C overnight, followed by second step avidin-alkaline phosphatase (Sigma-Aldrich) at 37°C for 1 h. Plates were developed using 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich). One volume of 3% (w/v) agarose was mixed with 4 volumes of 1.25 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 2-aminoo-2-methyl-1-propanol buffer (Sigma-Aldrich); then the mixture added to the plates. After color development, blue spots were counted manually using a dissecting microscope.

SYBR green real time PCR analysis

Purified B cells from HEL-Ig-transgenic mice were preincubated with 20 µg/ml LPS, and then stimulated with 20 ng/ml murine IL-2 (2 BD Pharmingen) and 1/200 dilution of HEL supernatant for 1 h as a positive control. Cells were lysed in RNA-Be RNA Isolation Solvent (TEL-TEST) for RNA isolation. Oligo(dT) primers and murine Moloney leukemia virus reverse transcriptase (all from Invitrogen Life Technologies) were used for preparation of cDNA following the manufacturer’s instructions. Primers used for quantitative real-time PCR were as follows: DUSP5 (forward, 5'-GACAGCCACACTGCTGCA CAT-3'; reverse, 5'-AGGAATGCAGCCTCCTTTCCTTCC-3'); β-actin (forward, 5'-GGCTGA AAGATGACCAGCAG-3'; reverse, 5'-TGGCT-3'GACCAGAGGACTACAG-3'). SYBR green real time PCR was performed on an ABI7700 sequence detection instrument (PE Applied Biosystems) following the manufacturer’s instructions.

*Abbreviations used in this paper: HEL, hen egg lysozyme; PBLs, phorbu dye; EGFP, enhanced GFP; AFC, Ab-forming cell; cIg, cytoplasmic Ig.
Results
Constant exposure to Ag inhibits LPS-induced plasma cell differentiation

To understand how Ag inhibits the differentiation of B cells in response to TLR4 stimulation, naive and anergic HEL-specific B cells (21, 37) were cultured for 4–5 days with LPS in the presence or absence of HEL Ag. Plasma cell differentiation is driven by expression of Blimp-1 and extinction of Pax-5 transcription factors, thus decreasing expression of surface Ig, B220, and CD72 and increasing syndecan-1, J chain, and intracellular Ig (43–45). Based on each of these molecular markers, the continuous presence of HEL Ag inhibited LPS-induced plasma cell differentiation of both naïve and anergic HEL-binding B cells (Figs. 1, A–D, 2E, and 4A). As shown in Fig. 2E, constant binding of Ag blocked the appearance of Blimp-1, the master transcriptional regulator of plasma cell differentiation. The inhibitory HEL dose response paralleled that required to tolerize these B cells in vivo (46), and inhibition was Ag specific given that the addition of HEL did not alter differentiation of nontransgenic B cells that lack HEL-specific BCRs (Fig. 1D). Inhibition of plasma cell differentiation was unrelated to cell proliferation. LPS induced equivalent proliferation in naïve and anergic B cells, and addition of Ag had no effect on LPS-induced proliferation of anergic B cells whereas it increased proliferation of naïve B cells as previously shown (37).

To distinguish whether Ag inhibited an early step in the differentiation program or was reversing a differentiation process already under way, Ag was added together with LPS or after a delay of 1, 2, or 3 days (Fig. 1E, filled symbols). CD138/syndecan-1 expression was used as a single cell reporter for Blimp-1 activity, since retroviral transduction of prdm1 deficient B cells has firmly shown that Blimp-1 is both necessary and sufficient to drive CD138/syndecan expression in LPS stimulated B cells (47). In the absence of HEL (open symbols), syndecan-1 expression was detectable on a small fraction of cells 2 days after stimulation with LPS, and the fraction of differentiated cells peaked on day 4. When HEL was present throughout the culture period, few plasma cells were formed. Delaying addition of HEL Ag until 1 day after stimulation with LPS was still almost fully effective at inhibiting differentiation, indicating that coincident LPS and Ag receptor signaling is not required to block initiation of an LPS-induced differentiation program. Delaying Ag receptor ligation until 2 or 3 days after LPS stimulation resulted in a less complete block and limited the frequency of syndecan-1-positive cells to those already differentiated at the time of Ag addition (compare open and filled symbols connected by line in Fig. 1E). Cells that had not yet expressed syndecan-1 apparently remain susceptible to the negative signal because addition of HEL at days 2 or 3 prevented further differentiation, which normally occurs between days 3 and 4 in the absence of Ag. These data support a model in which Ag receptor ligation acts just before differentiation to syndecan-1-positive stages but does not reverse the differentiation process.

To determine whether the block in differentiation requires continuous BCR ligation by Ag, B cells were stimulated with LPS and a pulse of HEL for 1, 2, or 3 days and then washed free of Ag and returned to culture with LPS alone (Fig. 1F). All cultures were analyzed on day 4. In cultures exposed to HEL Ag for just the first day, there was only partial inhibition in the fraction of cells that were syndecan-1 positive at day 4 (open symbols connected by arrows to the time when the pulse of HEL was stopped), when compared with cells exposed to HEL for the full length of the
culture (filled symbol on day 4). Pulsed exposure to Ag for the first 2 or 3 days of the 4-day culture, however, inhibited differentiation almost as much as constant exposure. These data indicate that the Ag signal that inhibits plasma cell differentiation requires persistent exposure to Ag and that the differentiation process requires more than 2 days to resume after a pulse of Ag.

**Constant Ag inhibits plasma cell differentiation via a calcium/calcineurin-independent and MEK/ERK mechanism**

Because anergic B cells selectively retain Ag-induced BCR signaling through the calcium-calcineurin-NFAT and ERK nuclear signaling pathways (41, 48), we tested whether or not these pathways account for the inhibition of LPS-induced plasma cell differentiation. Ionomycin treatment to induce calcium signaling comparable with that induced by Ag was unable to mimic the effect of Ag on plasma cell differentiation, nor could the calcineurin antagonist cyclosporin A relieve the inhibitory effect of Ag (Fig. 2, B–E). By contrast, the differentiation inhibiting effects of Ag could be reproduced by phorbol dibutyrate (PdBu; Fig. 2, B–E), an activator of the signaling pathway that proceeds through rasGRP to activate RAS, Raf, MEK, and ERK (49). PdBu had no effect on LPS-induced proliferation (not shown), and the concentration of PdBu needed to inhibit plasma cell differentiation (Fig. 2B) produced a level of ERK activation comparable with that induced by HEL Ag (Fig. 2, A and B).

The role of ERK signaling was further examined by pharmacological inhibition with the selective MEK antagonist, PD98059 (Fig. 3). Addition of 20 μM PD98059 inhibited Ag- or PdBu-induced phosphorylation of ERK, as well as inhibiting the pre-existing ERK activation in freshly isolated anergic B cells which already have HEL Ag docked on their BCRs in vivo (data not shown). In the absence of Ag, proliferation and differentiation of LPS-stimulated naive or anergic HEL-specific B cells was unaffected by addition of PD98059 as measured either by expression of the plasma cell marker, syndecan-1 (Fig. 3A), or by secretion of HEL-specific Ab in culture supernatant (Fig. 3B). By contrast, PD98059 reversed the inhibitory effect of HEL Ag on plasma cell differentiation (Fig. 3, A and B). In nontransgenic B cells, inhibition of plasma cell differentiation by constant BCR engagement with anti-IgM Ab was also reversed by PD98059 (Fig. 3C). Together with data in Fig. 2, these results indicate that continuous activation of the ERK kinase pathway by Ag is necessary and sufficient to prevent plasma cell differentiation in response to the TLR4 agonist LPS.

As an additional test for whether constant activation of the ERK pathway is sufficient to inhibit LPS-induced plasma cell differentiation, we used retroviral-mediated gene transfer to enforce expression in splenic B cells of a constitutively active form of MEK (MEK\(^*\); Ref. 42). The retroviral vector produced a bicistronic mRNA encoding both MEK\(^*\) and enhanced GFP (EGFP) which is a useful marker of transduced cells and a predictor of the relative bicistronic mRNA level in each cell. One day after stimulation with LPS in the presence or absence of HEL Ag, HEL-specific B cells were spin inoculated with supernatant containing MEK\(^*\)
Figure 3. MEK antagonism blocks BCR inhibition of LPS plasma cell differentiation. A and B, HEL-specific B cells were cultured as in Fig. 1 for 5 days with LPS in the presence or absence of HEL Ag or 20 μM MEK antagonist PD98059 (PD). Plasma cell differentiation was measured by staining for syndecan-1 (A) or by measuring secreted Ab in replicate culture supernatants (B). C: Nontransgenic spleen cells were cultured for 5 days with 20 μg/ml LPS, constant exposure to Ag was mimicked by including anti-IgM Abs, 20 μM PD98059 (PD) was added to indicated cultures, and supernatant Ab titers were measured by ELISA.

Dominant negative RAS interferes with BCR-mediated inhibition of plasma cell differentiation

BCR activation of the ERK pathway depends on RAS signaling, and ligation of BCRs on B cells activates p21<sup>ras</sup> within 1–2 min (50, 51). To examine the role of RAS signaling in BCR-mediated inhibition of plasma cell differentiation, we analyzed LPS-induced differentiation of spleen cells from transgenic mice expressing in B cells a dominant negative form of RAS (N17<sup>ras</sup>). Although pre-B cell development is delayed in adult N17<sup>ras</sup>-transgenic mice, peripheral B cells are present in normal numbers and proliferate equivalently to nontransgenic controls in response to LPS or anti-IgM Abs despite a 3- to 5-fold inhibition of BCR-induced MEK activation (40). LPS stimulation of splenic B cells from wild-type and dominant negative RAS mice caused ~35% of B cells to become syndecan-1<sup>high</sup> and CD72<sup>dull</sup> plasma cells (Fig. 5A). Constant ligation of the BCR with anti-IgM Abs blocked the differentiation of wild-type but not N17<sup>ras</sup>- B cells. In dose-response studies (Fig. 5B), 0.3 μg/ml anti-IgM Abs partially inhibited plasma cell differentiation of control B cells, and inhibition was complete with 10 μg/ml anti IgM. By contrast, the differentiation of B cells expressing N17<sup>ras</sup> was not affected unless doses of 10 μg/ml were used, and complete inhibition of syndecan-1 expression required 30–100 μg/ml anti-IgM Abs (Fig. 5B). Thus, an intact RAS pathway is required for BCR-induced inhibition of plasma cell differentiation.

IL-2/IL-5 signaling induces Dusp5 and reverses both Ag-induced ERK and inhibition of plasma cell differentiation

The preceding experiments showed that constant BCR engagement and signaling through RAS, MEK, and ERK inhibits plasma cell differentiation driven solely by TLR4. During an acute bacterial infection, B cells that recognize bacterial Ags are likely to receive a combination of TLR4 signals and cytokines from bacteria-specific helper T cells. The two best characterized B cell differentiation-promoting cytokines, IL-2 and IL-5, are well established to induce Blimp-1 and promote plasma cell differentiation and Ab production (7, 36, 43, 52). We therefore asked whether or not the ERK pathway served as a common integrator for BCR and T cell-derived signals regulating plasma cell differentiation. Naive HEL-specific B cells were cultured with LPS in the presence or absence of HEL Ag, with IL-2 and/or IL-5 added to the culture medium.

grams in Fig. 4A, and that a threshold level of MEK<sup>+</sup> is required for it to oppose plasma cell differentiation. Indeed, the intensity of EGFP was much lower in the few MEK<sup>+</sup> cells that had fully differentiated in LPS cultures, compared with the cells that remained undifferentiated. No such difference in EGFP was apparent between differentiated and undifferentiated cells transduced with control vector. Consistent with both these possibilities, differentiation was more completely inhibited by the combination of HEL throughout the culture period and transduced MEK<sup>+</sup> (Fig. 4A).

To confirm the above results, transduced GFP<sup>+</sup> cells from the cultures were sorted and HEL-specific Ab-forming cells (AFCs) were counted by ELISPOT assay. As shown in Fig. 4B, after 5 days of culture with LPS in the absence of Ag, ~50% of naive cells and 40% of anergic B cells transduced with control vector have the ability to produce Ab. The number of AFCs in HEL-treated cultures is one-half the number in non-HEL-treated cultures. The effect of Ag was mimicked in MEK<sup>+</sup>-transduced naive or anergic cells, and the combination of MEK<sup>+</sup> and Ag further suppressed the number of Ab-secreting cells. These results indicate that the MEK/ERK signaling pathway is sufficient to mediate the inhibitory effects of Ag on LPS-induced Ab secretion.
The combination of IL-2 and IL-5 completely reversed the inhibitory effect of constant Ag on LPS-induced plasma cell differentiation, as measured either by Ab secretion (Fig. 6A) or by Blimp-1 expression (Fig. 6B). Ag-induced ERK phosphorylation in LPS blasts was abolished in IL-2 and IL-5 exposed cells (Fig. 6C), indicating that the ERK signaling pathway is a common integrating point for the opposing effects of constant Ag binding and T cell-derived cytokines.

To explain how T cell-derived B cell differentiation factors might oppose BCR activation of ERK, we asked whether these cytokines might induce one of the dual-specificity phosphatases (DUSP) that oppose the action of MEK by removing activating serine/threonine and tyrosine phosphates from ERK. Microarray analysis of eosinophils has found that IL-5 treatment induces DUSP5 mRNA by 5-fold (53), whereas gene expression and biochemical studies found that IL-2 up-regulates DUSP5 mRNA and protein in T cells and inhibits ERK activity (54). DUSP5 can hydroyze proteins at both phosphotyrosine and phosphoserine/threonine residues, and recombinant DUSP5 can dephosphorylate phosphorylated ERK1 in vitro (55, 56). Real time PCR was used to measure Dusp5 expression in Ag- and cytokine-stimulated B cells (Fig. 6D). IL-2 and IL-5 or HEL exposure slightly increased Dusp5 mRNA compared with the LPS-induced basal expression; however, a combination of IL-2, IL-5, and HEL acted synergistically to increase Dusp5 gene expression to a level comparable with that in IL-2-stimulated EL4 T lymphoma cells.

To test whether Dusp5 induction is sufficient to explain cytokine reversal of BCR-ERK plasma cell inhibition, we expressed Dusp5 in naive B cells using a constitutively expressed bicistronic EGFP retroviral vector. Two days after transduction, GFP× cells were sorted by flow cytometry and HEL-specific IgM Ab-forming cells enumerated by ELISPOT assay. Percentage of AFC in each group is shown ±SEM, n = 4.

**Discussion**

In many species and cell types, the RAS/MEK/ERK pathway regulates cell differentiation. Whether it plays a positive or negative role in regulating differentiation may depend not only on the tissue type but also on the developmental stage of the cell and whether the receptor that activates it is able to induce sustained or transient ERK signaling (57). The RAS/MEK/ERK pathway promotes thymocyte-positive selection (58), antagonizes myocyte differentiation (59, 60), promotes neuronal differentiation (57), inhibits or promotes chondrogenesis depending possibly on duration (61), and promotes the initiation of adipocyte differentiation but inhibits its execution (62, 63). In B cells, the role of RAS is dependent on the developmental stage of the cell. In immature B cells, RAS promotes differentiation directly or indirectly (40, 64). We show here that the RAS/MEK/ERK pathway serves as a molecular switch to
The potential for chronic infection nevertheless renders the kinetics of Ag binding, on its own, an insufficient criterion for regulating plasma cell differentiation. Although bacterial Ags may continuously engage BCRs during chronic bacterial infection and potentially block antibacterial Ab responses, T cells are able to deliver B cell differentiation signals selectively to those B cells that have indeed bound, internalized and presented bacterial Ags. As shown here, the best characterized B cell differentiation factors from T cells, IL-2 and IL-5, provide a mechanism to override BCR inhibition of differentiation by inducing the ERK phosphatase, Dusp5, which inactivates the MEK-ERK pathway. It is interesting that these two B cell differentiation factors, and the more recently characterized and potent B cell differentiation factor IL-21 (65), share signaling through Stat3 and, in the case of IL-2 and IL-21, the common γ-chain. By contrast, other important T cell helper signals such as CD40L and IL-4 signal through different pathways and stimulate proliferation but not plasma cell differentiation. With respect to antibacterial Ab responses, it is notable that IL-5 plays an especially important role in mucosal Ab responses (7).

How does constant BCR signaling through ERK prevent plasma cell differentiation? The inhibitory effect can be firmly placed upstream of Blimp-1 expression, based on the following: 1) Blimp-1 expression is blocked (Figs. 2 and 6); 2) Ag can transmit the inhibitory effect as late as 2 or 3 days of LPS stimulation at the time when Blimp-1 expression is beginning and differentiation is under way (Fig. 1E); 3) forced expression of Blimp-1 reverses anti-IgM inhibition of LPS-induced differentiation (36). It is significant that differentiation resumes after Ag is removed (Fig. 1F) but only after a lag period of several days. This lag period may reflect the time taken for Ag-ERK signaling to decay, or the time for an inhibitor of Blimp-1 expression to be extinguished. Inhibitors of histone deacetylases promote Blimp-1 expression in primary B cells (66), and the histone deacetylase-interacting transcriptional repressor, Bcl-6, acts directly to repress the prdm1 gene encoding Blimp-1 and inhibit LPS-induced plasma cell differentiation (67). It is thus possible that BCR-ERK signaling inhibits Blimp-1 by maintaining Bcl-6 or its cofactors (see the penultimate paragraph in this section for an opposite effect in Ramos cells).

Alternatively, ERK modification of other pre-existing transcription factors may oppose Blimp-1 transcription. For example, Ets family proteins are activated by ERK phosphorylation on their pointed domain and can be recruited by BSAP/Pax-5 to form functional ternary complexes on a B cell-specific promoter (68). Because BSAP/Pax-5 can potentially inhibit Blimp-1 expression and overexpression of BSAP/Pax-5 in late B cells is sufficient to suppress differentiation into a high Ig-producing cell with plasma cell phenotype (69), ERK/Ets-1 could conceivably cooperate with BSAP/Pax-5 to oppose Blimp-1 induction. It will therefore be interesting in future studies to test whether BCR-ERK signaling represses Blimp-1 through Bcl-6 or via an independent pathway such as Ets-1.

As opposed to the inhibitory effects of BCR-ERK signaling, BCR-ERK may also stimulate plasma cell differentiation in specific settings. In contrast to the inhibition of differentiation in primary LPS-stimulated B cells here, BCR or phorbol ester induced ERK signaling in centroblast-like Ramos Burkitt lymphoma cells triggers the phosphorylation and degradation of Bcl-6 (70, 71). Two binding sites for fos/jun AP-1 heterodimers in the prdm1 promoter are implicated in promoting Blimp-1 expression in response to CD40L and IL-4 stimulation, and as targets for Bcl-6.

FIGURE 5. Dominant negative RAS interferes with the BCR signal that blocks differentiation. Splenocytes from transgenic mice expressing dominant negative N17ras in B cells, and from nontransgenic (Wt) controls, were cultured for 4 days with LPS in the absence or constant presence of anti-IgM Abs to mimic Ag. A. Representative profiles with 3 μg/ml anti-IgM. The percentage of differentiated plasma cells (syndecan-1/H11001) provides an explanation for the absence of GM1-reactive autoantibodies in serum Ig or bone marrow plasma cells despite the high frequency of circulating B cells with this BCR specificity (18, 19).

integrate inputs from Ag binding to BCR and from the best characterized differentiation factors from T cells to regulate LPS/TLR4-driven plasma cell differentiation (Fig. 7). These findings are especially significant because of the central role of LPS as a dominant immunogen in Ab responses to bacteria, its potential for antigenic mimicry with self Ags, and the uniquely potent ability of TLR4 to activate B cells via both MyD88 and TRIF signaling pathways.

The data in Figs. 1–5 show that sustained binding of Ag maintains a continuous intracellular signal through RAS, MEK and ERK to block LPS-induction of Blimp-1 and prevent the suite of plasma cell gene expression changes brought about by Blimp-1. Because self Ags are mostly invariant over time, a constant kinetic pattern of Ag binding is characteristic for BCRs that react with self Ags (Fig. 7). LPS can stimulate B cell proliferation and Ab secretion either after concentration on specific B cells by BCRs recognizing the polysaccharide or other linked epitopes, or as a polyclonal activator at higher concentrations such as those used here in vitro (3). Regardless of whether or not the BCR is reactive to a carbohydrate epitope on bacterial LPS (as in Guillain-Barré syndrome), another bacterial epitope, or the B cell is activated as a bystander, if the BCR also reacts with a self Ag, then chronic ERK signaling induced by the latter will normally suppress plasma cell differentiation induced by LPS (Fig. 7). By contrast, an acute pulse of Ag induced by the latter will normally suppress plasma cell differentiation? The inhibitory effect can be firmly placed upstream of Blimp-1 expression, based on the following: 1) Blimp-1 expression is blocked (Figs. 2 and 6); 2) Ag can transmit the inhibitory effect as late as 2 or 3 days of LPS stimulation at the time when Blimp-1 expression is beginning and differentiation is under way (Fig. 1E); 3) forced expression of Blimp-1 reverses anti-IgM inhibition of LPS-induced differentiation (36). It is significant that differentiation resumes after Ag is removed (Fig. 1F) but only after a lag period of several days. This lag period may reflect the time taken for Ag-ERK signaling to decay, or the time for an inhibitor of Blimp-1 expression to be extinguished. Inhibitors of histone deacetylases promote Blimp-1 expression in primary B cells (66), and the histone deacetylase-interacting transcriptional repressor, Bcl-6, acts directly to repress the prdm1 gene encoding Blimp-1 and inhibit LPS-induced plasma cell differentiation (67). It is thus possible that BCR-ERK signaling inhibits Blimp-1 by maintaining Bcl-6 or its cofactors (see the penultimate paragraph in this section for an opposite effect in Ramos cells).

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As opposed to the inhibitory effects of BCR-ERK signaling, BCR-ERK may also stimulate plasma cell differentiation in specific settings. In contrast to the inhibition of differentiation in primary LPS-stimulated B cells here, BCR or phorbol ester induced ERK signaling in centroblast-like Ramos Burkitt lymphoma cells triggers the phosphorylation and degradation of Bcl-6 (70, 71). Two binding sites for fos/jun AP-1 heterodimers in the prdm1 promoter are implicated in promoting Blimp-1 expression in response to CD40L and IL-4 stimulation, and as targets for Bcl-6.
FIGURE 7. Schematic illustrating the switch function of ERK signaling for controlling plasma cell differentiation between B cells with BCRs that recognize only an infectious bacterial epitope and B cells that recognize both a bacterial and a host (self) epitope. In acute infections, bacteria-specific B cells and B cells that bind both a bacterial and a self Ag will be stimulated by bacterial LPS via TLR4, activating NFκB and cell proliferation. Bacteria-specific B cells will experience a transient pulse of BCR signaling, indicated by dashed lines, to ERK and to NFκB, with the latter synergizing with TLR4 signals to augment clonal expansion. Because acute infectious bacterial Ags are typically present in small quanta, transient BCR-ERK signaling will allow plasma cell differentiation and anti-bacterial Abs to be secreted. Presentation of bacterial peptides to T cells will enhance secretion of these Abs by inducing Dusp5 and negating BCR-ERK inhibition of differentiation. Self-cross-reacting B cells will experience constant BCR-ERK signaling that inhibits LPS-induced Blimp-1 expression and plasma cell differentiation. Desensitization of BCR signaling to NFκB in self-reactive cells also eliminates synergistic signals from the BCR for clonal expansion and for effective collaboration with T cells. The combination of these factors prevents bacterial antigenic mimicry from triggering secretion of autoantibodies.
repression (72, 73). However, no role for fos could be detected in LPS-induced differentiation (73), and the chronic ERK signaling induced by HEL in anergic B cells does not increase fos or jun mRNAs despite elevating mRNAs for SRF target genes such as Egr1 (48). As noted above, IL-2 and IL-5 induce Blimp-1 expression and differentiation by activating Stat3 (74), and the prm1 gene contains Stat3-inducible elements that are separate from the Bcl-6-repressive elements (67, 75). It is interesting that whereas Stat3 tyrosine phosphorylation and nuclear translocation is triggered by IL-2 receptor signaling, Stat3 trans-activating activity is enhanced by TCR signaling to activate serine 727 phosphorylation by ERK (76). BCR-ERK signaling to Stat3 may explain the switch from an inhibitory effect of BCR-ERK signaling on its own to a synergetic induction of Blimp-1 by the combination of IL-2, IL-5, and Ag (Fig. 6).

The findings here establish the RAS/MEK/ERK signaling pathway as a central switch controlling TLR4-induced plasma cell differentiation, integrating opposing inputs from BCR and T cell cytokines. This inhibitory switch could explain the requirement for differentiation, integrating opposing inputs from BCR and T cell cyto
tokines. This inhibitory switch could explain the requirement for IL-5 for autoantibody secretion in anti-erythrocyte-transgenic mice (77) and Lyn-deficient mice (78). The architecture of this switch highlights many points where inherited variation could weaken the BCR-ERK pathway or enhance the IL-2/IL-5/Dusp5 pathway to allow bacterial infections to trigger secretion of autoantibodies, as seen in <1 in 1000 C. jejuni infections that proceed to Guillain–Barre syndrome.

Acknowledgments

We thank Drs. Roger Perlmutter and Brian Irani for the generous gift of the dominant negative RAS-transgenic mouse, Dr. Natalie Ahn for the generous gift of the activated MEK* cDNA, Dr. Garry Nolan for Phoenix breeding of the transgenic mice, and Aisling Murtagh and Suzanne Ewing for the staff at the Australian Phenomics Facility and Australian Cancer Research Foundation Genetics Laboratory for expert care and breeding of the transgenic mice, and Aisling Murtagh and Suzanne Ewing for expert genotyping.

Disclosures

The authors have no financial conflict of interest.

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