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Jannek Hauser, Jiyoti Verma-Gaur, Anders Wallenius and Thomas Grundström

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Initiation of Antigen Receptor-Dependent Differentiation into Plasma Cells by Calmodulin Inhibition of E2A

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Differentiation of B lymphocytes into Ab-secreting plasmablasts and plasma cells is Ag driven. The interaction of Ag with the membrane-bound Ab of the BCR is critical in determining which clones enter the plasma cell response. However, not much is known about the coupling between BCR activation and the shift in transcription factor network from that of a B cell to that of ASC differentiation. Our genome-wide analysis shows that Ab-secreting cell differentiation of mouse B cells is induced by BCR activation through very fast regulatory events from the BCR. We identify activation of IFN regulatory factor-4 and down-regulation of Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Spi-B gene expression as immediate early events. Furthermore, the transcription factor E2A is required for the rapid key down-regulations after BCR activation, and the Ca2+ sensor protein calmodulin has the corresponding regulatory effect as BCR activation. Moreover, mutants in the calmodulin binding site of E2A show that Ca2+ signaling through calmodulin inhibition of E2A is essential for the rapid down-regulation of immediate early genes after BCR activation in initiation of plasma cell differentiation.

DNA microarray analysis

The analysis of gene expression changes in B cells upon BCR activation was performed using the Illumina BeadChip system. For in vitro transcription amplification, 200 ng of RNA was used with the Illumina RNA Amplification Kit (Ambion). Amplified RNA (1.5 μg) was hybridized to the Sentrix MouseRef-6 Expression Beadchip (Illumina) containing 47,667 probes. The primary data were collected from the BeadChips using the manufacturer’s BeadArray Reader and analyzed using the supplied scanner software. Data normalization was performed by cubic spline normalization using Illumina’s Beadstudio v3 software. Clustering of certain selected genes was done using cluster 3 software, and data were visualized using tree view v1.2 software. All raw CEL dataset files are available at www.ncbi.nlm.nih.gov/geo (accession no. GSE15606).

Western blot

Nuclear extracts were prepared, as previously described (13), and Western blot was performed using the WesternBreeze immunodetection system (In-vitrogen), according to the manufacturer’s instructions, and using Abs listed in supplemental Materials and Methods.

Plasmids and viruses

The pcDNAI-based expression vector for mammalian CaM, the short hairpin RNA (shRNA) that interferes with E12/E47-specific human RNA, the EBV-based shuttle vector pMEP4 derivatives encoding wild-type and CaM-resistant m847 and m8N47 mutants of mouse E12, and the murine stem cell virus (MSCV)-internal ribosome entry site-GFP-based retroviruses have all been described previously (12–14).

Injection of mouse B lymphocytes

Retrovirus concentrated by centrifugation was added with 5 μg/ml polybrene to 0.5 × 10⁷ purified B cells after activation with LPS plus IL-4 for 14 h (24 h in Fig. 6). After a 12-h incubation, the infection was repeated for 12 h, followed by incubation for a further 22 h postinfection in fresh complete medium with the stimulants to allow for expression of GFP and E12 or CaM. In experiments after activation with CD40L plus IL-4, the medium was supplemented with LPS (2.5 μg/ml) during retroviral infection incubations to improve infection efficiency. Where indicated, anti-mouse IgM was added for the indicated time. Intracellular immunostaining of harvested cells was done with 2% paraformaldehyde and ethanol, as previously described (15), using Abs as listed in Materials and Methods. Stainings were done for at least 30 min at room temperature in the dark. Flow cytometry was with a FACSCalibur instrument and analysis with CellQuest software (BD Biosciences).

Cell culture and transfections

The human B cell lymphoma line DG75 (16) was maintained in RPMI 1640 medium supplemented with 5% FCS and antibiotics. DG75 cells were transfected by electroporation with 2 μg of CaM expression plasmid or the empty pcDNAI/amp vector. Eight hours later, live cells were separated using Lymphoprep (Axis-Shield), and 12 h later anti-IgM was added to one-half of the cells, followed by continued culture for 3 h. DG75 cells transfected to express empty pMEP4 derivative, or pMEP4 derivative encoding the shRNA, E12, or m847 or m8N47 mutant of E12, were selected by hygromycin B (Roche) for 5 days, as described previously (12). The BCR of DG75 was activated by stimulation of 1 × 10⁶ cells for 3 h, unless otherwise specified, with 5 μg of goat Fab(ab’); anti-human IgM (Southern Biotechnology Associates) in 1 ml of complete RPMI 1640 medium supplemented with 5% FCS.

Real-time RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), and real-time PCR analysis was performed, as previously described (12), using GAPDH as an internal control. The specificity of the real-time PCR was analyzed by melt-curve analysis, as described by the manufacturer, and the sizes of the PCR products with the different primer pairs were verified by 2.5% agarose gel electrophoresis. Some of the primer pairs used have been described previously (12), and the additional RT-PCR primer pairs used are listed in supplemental Materials and Methods.

Results

Very fast gene-regulatory events upon activation of the BCR

To analyze whether ASC differentiation is Ag driven in an in vitro cultivation system, B cells purified from mouse spleen were first activated using LPS and IL-4, and the effect of activation of the Ag receptor with anti-IgM after 2 days of cultivation was analyzed. To examine ASC differentiation, FACS analyses were performed after immunostaining for syndecan-1 (CD138), a marker for plasma blasts and plasma cells. Compared with the cells not given BCR activation, BCR activation for 1 day produced many more plasma blasts and plasma cells. Compared with the cells not given BCR activation, syndecan-1 expression increased between 2.5 and 3 times in this increased population of
highly syndecan-1-expressing cells. After a second day of BCR activation, the highly syndecan-1-expressing cells increased in size, a change expected for differentiation into ASC cells. In the control without BCR activation, the frequencies of large and strongly syndecan-1-expressing cells changed little (Fig. 1A). Thus, activation of the Ag receptor increases plasmablast/plasma cell differentiation in this system. The increase in frequency of syndecan-1 high cells and their syndecan-1 expression level at day 1 and the shift to larger highly syndecan-1-expressing cells at day 2 were also obtained when stimulating the BCR of cells activated with 4-fold lower levels of LPS and IL-4 (Fig. 1B).

In the germinal center, the Ag receptor works with Th cells to determine selection of B cells for plasma cell differentiation, and CD40L (CD154) is the most important cytokine from the Th cells (2, 17). Therefore, we analyzed the effect of activation of the BCR with anti-IgM also when cultivating the splenic mouse B cells with CD40L instead of LPS together with the IL-4. This condition also resulted in a profound increase in large and highly syndecan-1-expressing cells when the BCR was stimulated (Fig. 1C). The increase in large and highly syndecan-1-expressing cells by stimulating the BCR was even stronger when the levels of CD40L and IL-4 were reduced 4- or 10-fold (Fig. 1D, and data not shown).

Thus, Ag receptor stimulation increases plasmablast/plasma cell differentiation both in Th cell-independent and Th cell-dependent differentiation and over a range of concentrations of LPS, CD40L, and IL-4.

**BCR activation results in a large number of very rapid transcription changes**

To search for rapid changes in gene expression following BCR activation, we performed DNA microarray analysis of activated splenic B cells with and without anti-IgM treatment for 3 h. Cells activated with CD40L and IL-4 were used because this activation resulted in the largest increase in ASC differentiation (cf Fig. 1, A and B with Fig. 1, C and D) and because most Ab responses are Th cell dependent, mimicked by the main cytokine CD40L. The change in expression for each gene was computed as a ratio of expression in anti-IgM-treated B cells vs untreated control B cells. Using the Illumina Mouse Beadchip system, the expression of 31,492 genes was examined. Dendrogram analysis of these genes demonstrated a high degree of reproducibility between the three mice analyzed (data not shown). The expression of a remarkably large set of genes differed significantly (≥1.5-fold change, p < 0.05) between the BCR-stimulated and nonstimulated B cells: 2,259 genes were up-regulated, and 2,345 genes were down-regulated. The cluster plots of certain genes with a coupling to B cell lineage (18). The finding that ~7% of the genes were up-regulated and 7% were down-regulated within 3 h of BCR activation (supplemental Table S1) is comparable to the reported difference in expression of 15% of the genes (≥2-fold) between purified mouse germinal center B cells and plasma cells (19). Thus, a significant part of the change in mRNA level happens already within 3 h for most of the genes that change expression in differentiation from a B cell to an ASC.

To study the earliest Ag receptor-driven transcriptional changes in splenic B cells, the mRNA levels were followed by quantitative real-time PCR after the BCR activation, which has been correlated with the commitment to the plasma cell lineage (18). The finding that ~7% of the genes were up-regulated and 7% were down-regulated within 3 h of BCR activation (supplemental Table S1) is comparable to the reported difference in expression of 15% of the genes (≥2-fold) between purified mouse germinal center B cells and plasma cells (19). Thus, a significant part of the change in mRNA level happens already within 3 h for most of the genes that change expression in differentiation from a B cell to an ASC.

**FIGURE 2.** Rapid differential gene expression upon BCR activation. Cluster plots of selected important genes differentially expressed during 3 h of anti-IgM stimulation of the BCR of B lymphocytes from mouse spleen activated with CD40L (200 ng/ml) plus IL-4 (5 ng/ml). Total RNA was isolated from B cells of three independent mice with and without the stimulation and amplified and hybridized on Illumina BeadChips. The fold differential expression of genes in the individual mice is color coded, with increases marked in red and decreases marked as green. The functional classification of genes was done using gene ontology information provided by Illumina, and selected genes are included.
shown). However, this was not the case for all genes. The mRNA level for Pax5, a key transcription factor for B cell identity and repressor of the ASC differentiation program, was significantly reduced already within 30 min of BCR stimulation. The reduction was $\sim$3-fold within 1 h and 4- to 5-fold within 3 h (Fig. 3A). The mRNA level of Bcl-6, another repressor of ASC differentiation, also decreased significantly within 30 min of BCR stimulation, and this decrease was also $\sim$3-fold within 1 h and 4- to 5-fold within 3 h (Fig. 3A). The level of mRNA for MITF, one more repressor of ASC differentiation, was reduced by $\sim$50% within 30 min of the BCR stimulation (Fig. 3A). The mRNA levels of the Ets family members Ets-1, Fli-1, and Spi-B, all implicated in maintenance of B cells (1, 3, 7, 8, 11), were also significantly reduced within 30 min of the BCR stimulation (Fig. 3A). Their mRNA levels were reduced between 2 and 3 times within 1 h, and they decreased further within 3 h (Fig. 3A). The reduction was especially pronounced for Ets-1, which had only 18% of the mRNA remaining after 3 h. Thus, down-regulation of the mRNA for several repressors of ASC differentiation are very early regulatory events that are initiated within less than 30 min and carried far within 1 h when activating the Ag receptor of splenic B cells. The mRNA levels for IRF-4 and Blimp1, important regulators of ASC differentiation and repressors of Pax5 and Bcl-6, increased relatively fast, although perhaps slightly slower than the six down-regulations. These mRNA increases reached 2-fold within less than 2 h (Fig. 3A).

The very fast reductions of the mRNA levels for several repressors of ASC differentiation could be through inhibition of the transcription, provided that the mRNA is constitutively short-lived, or, alternatively, through anti-IgM-induced degradation of the mRNA. Therefore, the effect of BCR activation was compared with that of an inhibitor of transcription, actinomycin D. The mRNA level for each of Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Spi-B was found to fall with approximately the same rate as after anti-IgM treatment also after actinomycin D treatment (data not shown). Thus, the mRNAs of these genes are all constitutively short-lived, and the fast reduction in mRNA level after Ag receptor stimulation...
appears to be through inhibition of transcription for each of these genes. To determine whether the rapid reductions in the mRNA of these genes and the increase in Blimp1 mRNA after BCR activation resulted in corresponding changes at the protein level, we performed Western blot analyses. A typical Western for each protein is shown in Fig. 3B. All reductions in mRNA levels led also to reductions in the protein levels, although somewhat delayed compared with the mRNA levels. At 30 min, when the mRNA levels were already reduced (Fig. 3A), the Pax5, Bcl-6, MITF, Ets-1, and Fli-1 protein levels remained at 105 ± 10, 90 ± 21, 100 ± 15, 95 ± 3, and 92 ± 9%, respectively (n = 3). However, the levels of these proteins did decrease to 85 ± 9, 76 ± 27, 51 ± 8, 93 ± 17, and 80 ± 8%, respectively, after 1 h, and to 56 ± 12, 62 ± 18, 38 ± 4, 73 ± 7, and 48 ± 5%, respectively, after 2 h (n = 3). In contrast, the increase in Blimp1 protein was several hours delayed compared with the increase in Blimp1 mRNA, indicating the presence of posttranscriptional regulation of the protein level after the BCR activation for this protein.

We analyzed the time courses of the effects of activation of the BCR also after combining the IL-4 treatment of the splenic mouse B cells with CD40L instead of LPS. The BCR stimulation resulted in the corresponding down-regulation of Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Spi-B mRNA, as well as a reduction in MTA3 and BACH2 and up-regulation of IRF-4 and Blimp1 mRNA when using CD40L instead of LPS in the activation (Fig. 3C). Notably, the decrease in Bcl-6 mRNA was more pronounced when using CD40L. This agrees with our observation that CD40L increases Bcl-6 mRNA expression to a higher level than LPS does (data not shown), and with Bcl-6 being a strongly expressed master regulator of germinal centers whose formation depends on Th cells and CD40L (2). The effects of BCR stimulation on the expression of the 10 genes were similar after combined stimulation with CD40L and LPS (at 4-fold reduced levels) together with IL-4 (data not shown). Thus, Ag receptor stimulation results in the rapid key regulatory changes both in Th cell-independent and Th cell-dependent ASC differentiation.

The early regulatory events upon activation of the BCR depend on Ca²⁺ signaling and CaM

Activation of Ag receptor leads to formation of a large complex of proteins below the receptor that rapidly increases the intracellular Ca²⁺ concentration and results in a combination of Ca²⁺ signaling and a cascade of phosphorylations (21). To investigate whether Ca²⁺ signaling and/or serine protein kinases were essential for the effects of BCR stimulation on the expression of Pax5, Bcl-6, MITF, Ets-1, Fli-1, IRF-4, Spi-B, and Blimp1 mRNA, the activation with anti-IgM was performed in the presence of various inhibitors of the signaling pathways. The Ca²⁺ chelator BAPTA-AM completely blocked the effect of BCR stimulation on the mRNA level of all the eight genes studied, and inhibition of L-type Ca²⁺ channels with Nifedipine or inositol 1,4,5-triphosphate receptor Ca²⁺ channels with TMB-8 partially or completely blocked the effect, especially when combined (supplemental Fig. S1). Thus, Ca²⁺ signaling was essential for the effect of BCR stimulation on the expression of mRNA for each of these transcription factors. However, the effects of inhibitors of Ca²⁺/CaM-dependent protein kinase (CaMK) or Ca²⁺/CaM-dependent phosphatase calcineurin varied for the different proteins and were in most cases smaller or absent (supplemental Fig. S1). The MAPK inhibitor PD98059 blocked the reductions in Pax5, Bcl-6, MITF, and Spi-B mRNA and the increase in IRF-4 mRNA, and it partially blocked the increase in Blimp1 mRNA and the reduction in Ets-1 and perhaps also Fli-1 mRNA (supplemental Fig. S1). This suggests that, in addition to the Ca²⁺ signaling, MAPK signaling is important to different degrees for the BCR-induced changes in expression of all or most of the analyzed genes. The protein kinase C (PKC) inhibitor bisindolylmaleimide had little effect compared with Ca²⁺ chelator or Ca²⁺ channel blockers on the reductions in Pax5, Bcl-6, MITF, and Ets-1 mRNA and the increase in Blimp1 mRNA after BCR stimulation, whereas Fli-1 and Spi-B reductions and IRF-4 induction were affected by both types of treatment (supplemental Fig. S1).

Both stimulated splenic B cells and the easily manipulated human B cell lymphoma line DG75 (16) were used to investigate the Ca²⁺ signaling-dependent effects of BCR activation. As in stimulated splenic B cells, activation of the BCR with anti-IgM rapidly reduced the levels of Pax5, Bcl-6, Ets-1, and Fli-1 mRNA and increased the level of Blimp1 mRNA in the B cell line (Fig. 4A). MITF mRNA could not be detected in this cell line, and Spi-B expression was not inhibited (data not shown). The cause of these defects in MITF and Spi-B is unknown, but they might be coupled to the lymphoma phenotype of this cell line. The mechanism of IRF-4 induction was not analyzed because IRF-4 is induced by NF-κB (2) and the main Ca²⁺/CaM sensor protein CaM and CaMK II are essential for NF-κB activation after Ag receptor activation (14, 22).

To examine whether CaM could affect the expression of the Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Blimp1 genes, the effects of its overexpression were investigated. The efficiency of the CaM overexpression system in DG75 cells has been verified previously by Western blot analysis (12). The overexpression of CaM reduced the Pax5, Bcl-6, and Fli-1 mRNA levels ~2-fold and the Ets-1 mRNA level ~3-fold compared with vector control, and CaM increased the level of Blimp1 mRNA ~2-fold (Fig. 4B). The effects of overexpression of CaM were also analyzed in LPS- and IL-4-activated splenic B cells at the protein level by FACS. The cells were infected with retrovirus-expressing CaM, followed by an internal ribosome entry site and GFP. The FACS plots of a representative experiment with Pax5 are shown in supplemental Fig. S2A. The signals obtained with Pax5 Ab were up to 100-fold higher than without primary Ab, and 30–60% of the cells were infected. Analysis of the FACS plots showed that CaM overexpression not only reduced Pax5 of GFP-positive cells, but also had some effect on noninfected cells of the same plot when compared with cells not exposed to CaM-overexpressing cells. This reproducible finding is probably a bystander effect, i.e., that infected cells can signal to their neighboring cells. It is notable that Ca²⁺ and MAPK signaling pathways, both of which regulate Pax5 and the other transcriptional regulators studied in this work, are involved in production of bystander effects on neighboring cells (23). To avoid any influence of bystander effects on the results, the effects of overexpressions on Pax-5 and the other regulators were compared with vector control infection and not with noninfected cells of the same FACS plot. The results of all experiments are summarized in Fig. 4C. As seen in the figure, up to 2-fold decreases in Pax5, Bcl-6, and MITF and increase in Blimp1 were obtained upon BCR activation of uninfected control samples, a finding that agrees with our Western blot results (Fig. 3B). The CaM overexpression system, previously verified by Western blot analysis (12), reduced Pax5, Bcl-6, and MITF, and increased Blimp1 protein levels also in the primary splenic B cells (Fig. 4C). Effects of overexpression of CaM were also analyzed after activation with CD40L and IL-4 (Fig. 4D and supplemental Fig. S2B), and this resulted in ~2-fold decreases of Bcl-6, Ets-1, and Fli-1 expression. The level of Bcl-6 protein was higher at this condition compared with in presence of LPS plus IL-4, and the reductions in Bcl-6 protein on CaM overexpression and after BCR activation were larger (Fig. 4, C and D), findings that agree with our observations that CD40L plus IL-4 activate Bcl-6 mRNA much more than LPS plus IL-4 (data not shown). In summary, Fig. 4, B–D, shows that overexpression of CaM has the corresponding effect as BCR activation on Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Blimp1 expression.
the BCR did not significantly affect the level of E2A mRNA for at least 12 h in mouse splenic B cells (supplemental Fig. S3). Id proteins, which are inhibitors of DNA binding of E proteins, participate at several regulatory steps in B cell lineage development (26, 27). However, none of the Id proteins showed a large increase in mRNA level during 5 h of BCR activation that could explain the pronounced rapid decreases in Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Spi-B or the increase in Blimp1 mRNA in mouse splenic B lymphocytes (supplemental Fig. S3) or DG75 B cells (12). In addition, no significant change is seen in the expression level of any of the Id proteins in DG75 B cells (12). Furthermore, BCR activation does not decrease the amount of E2A that can bind to DNA in the absence of Ca^{2+} (12); therefore, no decrease in the level of E2A or increased inhibition of its DNA binding by Id proteins mediates the rapid transcriptional effects of BCR stimulation.

We have reported a series of mutants in the basic DNA and CaM-binding sequence of the E2A isoform E12 that through combinations of mutations are resistant to CaM to different extents (13). To investigate the possible role of Ca^{2+}/CaM inhibition of E2A in the regulation of Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Blimp1, we used the most CaM resistant of these, the m847 mutant, which has three amino acid substitutions. This mutant, however, is also resistant to Id proteins, whereas a second mutant used, m8N47, is sensitive to Id proteins and almost as resistant as the m847 mutant to CaM (12). We used a hygromycin-selectable EBV-based shuttle vector that stably directs synthesis of shRNA that interferes with human E2A mRNA. It reduces expression of both E2A mRNA and E2A protein over 10-fold in DG75 B cells (12). This shRNA expression plasmid reduced expression of Pax5 and Ets-1 mRNA in DG75 B cells by ~2-fold, Bcl-6 by ~1.3-fold, and Fli-1 by 3-fold, whereas Blimp1 expression was increased by ~2-fold (Fig. 5A), showing that the level of expression of these genes is E2A dependent. The reduction in E2A and the resulting effects on the expression of these genes were specific, because the shRNA expression plasmid did not affect the expression of a number of other BCR-regulated genes that have not been reported to be E2A regulated (Fig. 5A) (12) (data not shown).

BCR activation with anti-IgM reduced Pax5, Ets-1, Bcl-6, and Fli-1 mRNA 1.3- to 3-fold and increased Blimp1 mRNA 2- to 3-fold in nontransfected DG75 cells or in the presence of vector control plasmid (Fig. 5A). In contrast, expression of these genes was approximately at the level of the anti-IgM-treated cells transfected with vector control both with and without anti-IgM treatment when the shRNA expression plasmid was present (Fig. 5A). Thus, BCR activation had no further effect on the expression of any of these genes when E2A expression was inhibited (Fig. 5A). This loss of effect of anti-IgM was specific, because expression of the shRNA against E2A did not influence the effect of anti-IgM for any control gene analyzed that was induced or inhibited by BCR activation and not E2A regulated (Fig. 5A) (12) (data not shown).

The decrease in E2A mRNA was reversed by cotransfection with expression plasmid for the mouse E12 isoform of E2A, because the expressed shRNA does not interfere with mouse E2A. This complementation did not significantly change the expression of any control gene, again confirming that they are not E protein regulated (Fig. 5A) (12) (data not shown). This complementation fully restored both the levels of expression of Pax5, Bcl-6, Ets-1, Fli-1, and Blimp1 mRNA and the sensitivities to anti-IgM treatment (Fig. 5A). Importantly, the sensitivity to BCR stimulation was completely lost when complementing with a CaM-resistant mutant of E12. Anti-IgM had no effect on expression of any of the genes upon complementation with either the m847 or the m8N47 mutant of E12 (Fig. 5A). The loss of the effects of BCR stimulation on these genes was attributed to the loss of CaM sensitivity and not...
to loss of sensitivity to an Id protein, because the losses of effect of anti-IgM were as complete with the m8N47 mutant that is only resistant to CaM as with the m847 mutant that is also resistant to Id proteins. This loss of effect of anti-IgM on the five genes was specific, because CaM-resistant m847 or m8N47 mutant of E12 did not affect the result of anti-IgM treatment for any control gene stimulated or inhibited by BCR activation, including IRF-4 (Fig. 5A) (12) (data not shown).

To examine further whether the Ca$^{2+}$/H11001 signaling from the BCR affected expression of Pax5, Bcl-6, MITF, Blimp1, Ets-1, and Fli-1 through CaM-mediated inhibition of E2A, we performed FACS analyses of primary splenic B cell cultures infected with retrovirus expressing wild-type or CaM-resistant E12 (Fig. 5, B and C, and representative FACS plots in supplemental Fig. S4). We found a clear difference between primary splenic B cells infected with wild-type E12 that showed Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Blimp1 expressions sensitive to anti-IgM treatment and cells infected with CaM-resistant m847 or m8N47 mutant of E12 that showed clearly anti-IgM-resistant expression of these genes (Fig. 5, B and C). The decrease in Bcl-6 expression upon BCR activation after infection with retrovirus expressing wild-type E12 was larger in cells activated with CD40L and IL-4 than with LPS and IL-4 (Fig. 5, cf B and C), a finding that agrees with the results from noninfected or vector-infected cells (Fig. 4, C and D). This larger decrease is probably due to the much higher initial activation of Bcl-6. In summary, the inhibitions of Pax5, Bcl-6, MITF, Ets-1, and Fli-1 expression and the activation of Blimp1 expression after BCR activation all depend on CaM sensitivity of E2A.

Plasma cell differentiation is regulated by CaM inhibition of E2A

To investigate whether plasma cell differentiation is regulated by CaM and dependent on CaM sensitivity of E2A, we used the in vitro system for plasma cell differentiation (Fig. 1) and infected the purified splenic B cells with retrovirus expressing either CaM, wild-type E12, or CaM-resistant E12, followed by staining for the plasma cell marker syndecan-1 and gating for GFP-positive cells (infected cells) in the FACS analysis. Two days of anti-IgM stimulation of the BCR of B cells activated with LPS and IL-4 increased the frequency of large highly syndecan-1-expressing cells in the vector control infection (Fig. 6A), similarly to the results illustrated in Fig. 1. The generally higher frequencies of large syndecan-1-positive cells in Fig. 6 occur because gating for infected cells enriches for large highly syndecan-1-expressing cells, presumably as a combined effect of more efficient infection of cells prone to become larger and more syndecan-1 expressing together with cells becoming more active and thereby larger due to the infection. Nevertheless, the effect of BCR stimulation on the frequency of large highly syndecan-1-expressing cells was even larger in Fig. 6A than in Fig. 1 (14.2% (33.7-19.5%) vs 6.4% (7.5-1.1%)). Importantly, overexpression of CaM resulted in an increase in large highly syndecan-1-expressing cells even without anti-IgM treatment. This increase, approximately as large as upon stimulation of the BCR (Fig. 6A), shows that overexpression of CaM has a corresponding positive effect on plasma cell differentiation as BCR activation. This result agrees with our findings on

**FIGURE 5.** Loss of anti-IgM sensitivity of Pax5, Bcl-6, Ets-1, Fli-1, MITF, and Blimp1 expression by expression of CaM-resistant E12. A, E2A expression was reduced in DG75 B cells by a shuttle vector that expresses shRNA targeting both E12- and E47-splice forms of human E2A mRNA. Shown are the effects of the shuttle vector that expresses shRNA interfering with human E2A mRNA and complementation by expression of wild-type or mutant mouse E12 on the expression of the indicated mRNA. Where indicated, cells were treated with anti-IgM for 3 h before harvest. The expression levels in cells transfected with empty pMEP4 vector and not treated with anti-IgM were set at 100%. Results are mean ± SD (n = 3).
the effects of overexpression of CaM on the regulation of Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Blimp1 (Fig. 4, B–D). A corresponding effect of overexpression of CaM on the frequency of large highly syndecan-1-expressing cells was obtained also in cells grown in presence of CD40L and IL-4 (Fig. 6B). Cells grown with either LPS and IL-4 or CD40L and IL-4 and overexpressing wild-type E12 were clearly affected by BCR activation, and a ~1.5- to 2-fold higher frequency of large highly syndecan-1-expressing cells was observed, a frequency similar to the vector control-infected cells (Fig. 6). Importantly, in contrast to expression of the wild type, expression of a CaM-resistant E12, either the Id-resistant m847 mutant or m8N47 that is sensitive to the Id proteins, resulted in a reduced plasma cell differentiation both with and without BCR stimulation, and this reduction appeared to be slightly larger upon BCR stimulation (Fig. 6). In summary, overexpression of CaM stimulates plasma cell differentiation, and the efficiency of initiation of Ag receptor-driven plasma cell differentiation depends on CaM sensitivity of E2A.

Discussion

Plasmacytic differentiation is induced in response to appropriate signals to generate specific Abs upon Ag exposure. The BCR plays a key role, and the strength of the interaction with Ag is critical in determining which clones enter the plasma cell response (4–6). Additional signals participating in plasma cell differentiation include pathogen-associated molecular patterns, which signal through TLRs, and cytokines and other T cell signals in which CD40L is of special importance (2, 17, 28). A large number of studies have identified several transcription factors that have to be regulated through inhibition of E2A by Ca2+ signaling, because they were blocked by Ca2+ chelator or a combination of Ca2+ channel blockers, and expression of all analyzed genes was reduced by the Ca2+ sensor CaM. Furthermore, the inhibitions of expression by BCR activation depended on CaM sensitivity of E2A when analyzed at the mRNA level and/or at the protein level. The resistance of the expression of these genes to BCR activation when E2A is mutated does not exclude that the other E proteins, E2-2 and HEB, may also contribute to the expression and become CaM inhibited like E2A when the BCR is activated. However, the reduction in mRNA for the analyzed proteins by shRNA against E2A in the DG75 cells and the dominant effect of CaM-resistant E12 over all endogenous CaM-sensitive E proteins suggest that E2A is the dominant E protein regulating these genes. Thus, Ca2+ signaling leading to inhibition of E2A by Ca2+-loaded CaM is needed for many gene expression changes in initiation of plasma cell differentiation.

We found that the inhibitions of Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Spi-B gene expression all depend on Ca2+ signaling, because they were blocked by Ca2+ chelator or a combination of Ca2+ channel blockers, and expression of all analyzed genes was reduced by the Ca2+ sensor CaM. Furthermore, the inhibitions of expression by BCR activation depended on CaM sensitivity of E2A when analyzed at the mRNA level and/or at the protein level. The resistance of the expression of these genes to BCR activation when E2A is mutated does not exclude that the other E proteins, E2-2 and HEB, may also contribute to the expression and become CaM inhibited like E2A when the BCR is activated. However, the reduction in mRNA for the analyzed proteins by shRNA against E2A in the DG75 cells and the dominant effect of CaM-resistant E12 over all endogenous CaM-sensitive E proteins suggest that E2A is the dominant E protein regulating these genes. Thus, Ca2+ signaling leading to inhibition of E2A by Ca2+-loaded CaM is needed for many gene expression changes in initiation of plasma cell differentiation.

The very rapid and parallel reductions in the mRNAs for Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Spi-B by BCR stimulation, before reduction of the protein levels, strongly suggest that these mRNA reductions are not secondary to another change in expression. E2A functions in hematopoietic progenitor cells to activate expression of EBF and Pax5 and establishment of the program of B cell-specific gene expression (26, 31). The genes shown to be down-regulated through inhibition of E2A by Ca2+-loaded CaM could all be direct targets of E2A. However, it is presently not known whether they have an important E2A binding site in a regulatory DNA
sequence of the gene. Therefore, we cannot exclude that one or more of them are indirect E2A targets. The direct alternative is, however, the most likely, because the levels of mRNA were reduced by one-half within approximately the same time after BCR activation as after blocking RNA synthesis with actinomycin D. Thus, the limiting factor is the decay rates of the mRNA, and any delay before reduction in transcription is at most a few minutes. This fact strongly argues against the possibility that CaM inhibition of E2A down-regulates a hypothetical unknown primary gene, which subsequently would down-regulate these genes. Instead, this suggests that Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Spi-B are direct targets of E2A.

BCR stimulation increased IRF-4 and Blimp1 mRNA levels relatively fast, but perhaps slightly slower than the rapid transcription factor down-regulations. IRF-4 and Blimp1 are important regulators of ASC differentiation and repressors of Pax5 and Bcl-6 (1, 3, 9, 10), but IRF4 participates in plasma cell differentiation also independently of down-regulation of Bcl-6 (9). Induction of IRF-4 expression after BCR activation depends on NF-κB activation and not on inhibition of E proteins by Ca²⁺-loaded CaM. The increase in IRF-4 was sensitive to Ca²⁺ chelator, Ca²⁺ blockers, PKC inhibitor, and CaMKII inhibitor, findings that agree with reports that IRF-4 is induced by NF-κB (2) and that PKCβ and the main Ca²⁺-sensor protein CaM and CaMK II are essential for NF-κB activation after BCR activation (14, 22, 32). Furthermore, neither shRNA against E2A nor expression of CaM-resistant E12 changes the level of IRF-4 mRNA or the effect of BCR activation on this expression in DG75 B cells (12).

The increase in Blimp1 mRNA, and even more in Blimp1 protein, after BCR activation was slower than the down-regulations of several repressors of the ASC development. Nevertheless, the induction of Blimp1 mRNA and protein expression did, like the down-regulators of the repressors, depend on CaM sensitivity of E12. This can be explained by the rapid inhibition of Pax5 and Bcl-6, which are direct repressors of Blimp1 expression (28), through this mechanism. Furthermore, MITF is also rapidly inhibited through this mechanism, and MITF is an inhibitor of IRF4, which is an activator of Blimp1 expression (28). In addition to induction of Blimp1 expression secondary to relief of repression dependent on CaM sensitivity of E2A, Blimp1 induction could also be the result of a shift from CaM-sensitive E protein homodimers to a CaM-resistant E protein heterodimer with another basic helix-loop-helix transcription factor at an important gene-regulatory DNA element. Such a scenario would be analogous to the important Ca²⁺/CaM-dependent shift from E protein homodimers to E protein heterodimers with MyoD in muscle development (33).

In summary, we report that initiation of plasma cell differentiation is Ag driven through many very fast primary regulatory events from the BCR. Down-regulation of expression of many key transcription factor genes, including Pax5, Bcl-6, MITF, Ets-1, Fli-1, and Spi-B, occurs within 30 min, Ca²⁺ signaling leading to inhibition of E proteins by Ca²⁺-loaded CaM is needed for many of the gene expression changes in initiation of plasma cell differentiation.

Disclosures

The authors have no financial conflict of interest.

References