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Inflammatory signal induced IL-10 production of marginal zone B-cells depends on CREB

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ABSTRACT

IL-10 is a suppressive cytokine that has been implicated in the pathophysiology of autoimmune disorders and can be produced by different cell types such as regulatory B-cells. Our previous work showed that under inflammatory condition MZ B-cells differentiated into IL-10 producing cells and contributed to the downregulation of collagen-induced arthritis, while follicular B-cells failed to do so. Based on these observations, we aimed to investigate how inflammatory signals mediated through the BCR, TLR9 and IFN-γ receptors trigger IL-10 production in MZ B-cells but leave FO B-cells unresponsive. We particularly focused on the CREB transcription factor as it is involved in all three signalling cascades and analysed its contribution to IL-10 production. Our results demonstrate that the IL-10 production of MZ B-cells induced by the BCR, TLR9 and IFN-γ receptors is mediated by CREB. We showed that the activation of CREB is prolonged in MZ B-cells while the transcription factor only transiently phosphorylated in FO B-cells. The sustained phosphorylation of CREB is clearly associated with its prolonged binding to molecular partner CBP, whereas inhibition of their association decreased IL-10 production. We assume that sustained activation of CREB is required for IL-10 production by B-cells under inflammatory conditions.

1. Introduction

It is now well established that beyond the production of self-destroying autoantibodies B-cells have a negative regulatory role in immune responses during autoimmunity associated inflammation [1]. Through the production of immunomodulatory cytokines, including IL-10, a variety of regulatory B-cell subsets have been described [2–6].

In mice, marginal zone (MZ) B-cells are mostly found in the outer white pulp of the spleen between the marginal sinus and the red pulp. They primarily express polyreactive B-cell antigen receptors (BCRs), which recognize conserved molecular patterns that are often shared by foreign and autologous antigens [7–9]. Compared to follicular (FO) B-cells, MZ B-cells show higher expression of surface IgM, complement receptors CD21/35 and lipid-antigen-presenting molecule CD1d [9,10]. In addition to the BCRs, mouse MZ B-cells express high levels of TLRs (Toll-like receptors) including TLR9, which recognizes hypomethylated CpG motifs in bacterial DNA or chromatin complexes [11–13].

Besides, there is evidence that MZ B-cells contribute to the pathogenesis of different autoimmune diseases, including the mouse model of human rheumatoid arthritis, CIA (collagen-induced arthritis) by shuttle type II collagen into the follicles and initiate autoimmune response [14], MZ B-cells like other innate-like cells or transitional-2 B-cells are indeed a good source of regulatory IL-10 [4,15–17]. Gray and coworkers showed that systemically administered apoptotic cells given to mice at the time of inducing CIA, directed MZ B-cells to secrete IL-10, that led to a markedly reduced severity of joint inflammation and reduced titres of pathogenic anti-collagen antibodies [18]. In line with these observations our group has recently published that MZ B-cells expressed an elevated level of suppressive IL-10 during the remission phase of CIA, furthermore robustly secreted the cytokine in response to inflammatory stimuli [19].On the contrary, it was interesting to learn that FO B-cells using the same *in vitro* experimental setup that mimicked synovial inflammation didn't show significant sensitivity for IL-10 induction [19].

Motivated by these findings we aimed to investigate how inflammatory signals, mediated *in vitro* through the BCR, TLR9 and IFN-γ receptors, trigger IL-10 production in MZ B-cells but leave FO B-cells unresponsive. To simplify the complex signalling events initiated by

Abbreviations: BCR, B-cell antigen receptor; CIA, Collagen-induced arthritis; CREB, cAMP response element-binding protein; FO, follicular; IFN-γ, interferon-gamma; MZ, marginal zone; TLR9, Toll-like receptor 9

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these receptors we selected the CREB (cAMP response element-binding protein) transcription factor as a molecular intersection point between the three signalling cascades and analysed its activation following the BCR, TLR9 and IFN-γ receptor stimulation and its contribution to the IL-10 production of MZ B-cells.

Our results show that CREB is an essential transcription factor in the IL-10 production of MZ B-cells. Activation of the transcription factor through the BCR, TLR9 and IFN-γ receptors is sustained in MZ B-cells while CREB is only transiently phosphorylated in FO B-lymphocytes. Sustained phosphorylation of CREB correlates with its prolonged association with CBP, while inhibition of their association decreases IL-10 expression by MZ B-cells. Taken together, we assume that prolonged activation of CREB is needed for the induction of regulatory differentiation of B-cells under inflammatory conditions.

2. Materials and methods

2.1. Mice

DBA/1 mice were originally obtained from Charles River Laboratories International and maintained in a specific pathogen-free animal facility at the Eötvös Loránd University. All experimental procedures were in accordance with national regulations and were authorized by the ethical committee of the University.

2.2. Antibodies and reagents

The following antibodies were used: non-conjugated or fluorescently labelled goat anti-mouse IgM $F(ab')_2$ (Jackson Immunoresearch), fluorescent rat anti-mouse IL-10, CD19, CD21, CD23, B220 (Becton Dickinson Biosciences (BD)), phospho-p38, phospho-Akt, phospho-CREB, phospho-GSK-3 (Cell Signaling Technology), monoclonal β-actin (Sigma-Aldrich) and HRP-conjugated anti-mouse/rabbit IgG (DAKO). Other reagents used for cell cultures were recombinant mouse IFN-γ (R &D Systems), CpG-ODN1826 (CpG; InvivoGen), phorbol 12-myristate 13-acetate (PMA) and ionomycin from Sigma-Aldrich, monensin (BD GolgiStop) and BD Cytofix/Cytoperm solutions.

2.3. B-cell purification and cell cultures

For the gene expression analysis MZ B-cells were purified from the spleen of DBA/1 mice after fluorescent labelling splenocytes with antibodies against CD21, CD23 and using FACSAria III cell sorter (BD). MZ B-cells were identified as $CD23^{-}CD21^{high}$ cells and sorted accordingly with purity > 97% (Supplementary Fig. 1A). For *in vitro* cell cultures and for Western blot analysis MZ B-cells were negatively selected by MACS using the Marginal Zone and Follicular B Cell Isolation Kit from Miltenyi Biotec with purity > 90 and 85%, respectively (Supplementary Fig. 1B). Sorted cells were cultured at a density of 2×10^6 cells/ml in RPMI-1640 supplemented with 10% FCS, 50 μM β-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in the presence or absence of IFN-γ (50 ng/ml), anti-IgM (10 μg/ml) and CpG (1 μM). In those experiments where the CREB inhibitor (5 μM; KG-501, Sigma-Aldrich) was used, the inhibitor was added to the cell cultures 60 min before the addition of IFN-γ, anti-IgM and CpG. According to the experimental setup, cells were harvested at different time points and either stained for flow cytometry or collected in TRIzol Reagent (Thermo Fisher Scientific) for gene expression analysis, while culture supernatants were collected and used to perform IL-10-specific ELISA.

2.4. IL-10 detection by ELISA

The BD OptEIA™ mouse IL-10 ELISA kit was used to detect the secreted amount of IL-10 in culture supernatants of MZ and FO B-cells according to the manufacturer's instructions. Briefly, microwells were coated with the capture antibody and blocked with an assay diluent. Standard or samples were added into the wells and incubated for 2 h at RT. After washes, a working detector was added to each well. Following the addition of the substrate solution the reaction was stopped and absorbance was read at 450 nm by an ELISA reader (Thermo Electron, Multiskan Ex). The amount of IL-10 was calculated using the standard curve and the GraphPad Prism software.

2.5. Cell surface and intracellular flow cytometry

For the detection of intracellularly expressed IL-10, sorted and cultured (24 or 48 h) MZ or FO B-cells were washed and re-suspended in medium containing 50 ng/ml PMA, 500 ng/ml ionomycin and 2 μM monensin (BD) and incubated for an additional 6 h at 37 °C and 5% CO_2. After the incubation cells were washed three times then stained for CD19 at 4 °C for 20 min in the dark. After washing, cells were fixed with BD Cytofix/Cytoperm solution for 20 min at 4 °C, washed with BD Perm/Wash buffer and intracellularly labelled with IL-10 specific antibody according to the manufacturer's instructions. Labelled cells were acquired on FACSCalibur flow cytometer (BD) and data were analysed by FlowJo software (FLOWJO, LLC).

2.6. Western blotting

Western blot analysis on MZ and FO B-cells was performed as described previously [20]. Briefly, MACS sorted cells were treated simultaneously with IFN-γ (50 ng/ml), anti-IgM (10 μg/ml) and CpG (1 μM) for different times (0, 30, 60 min, 3, 6, 24 h). Lysates from 2×10^6 cells were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were immunoblotted using antibodies against phospho-CREB, phospho-GSK-3, phospho-Akt, phospho-p38 and β-actin (loading control). Bindings were revealed by a HRP-conjugated anti-mouse/rabbit IgG antibody (Dako) and ECL (Advansta, WesternBright) detection.

2.7. Measurement of intracellular Ca2+ by flow cytometry

Sorted MZ or FO B-cells were resuspended in RPMI-1640 culture medium supplemented with 10% FCS, 50 μM β-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium) at 5×10^6/ml density and were loaded with 5 μM Fluo-4 AM in the presence of 100 μg/ml Pluronic F-127 at 37 °C for 30 min. After loading, samples were washed for 30 min at 37 °C, then resuspended in 10 ml of complete medium. Pre-warmed (5 min at 37 °C) samples of 5×10^5 Fluo-4 loaded cells were treated simultaneously with the indicated dilutions (0.2x, 0.1x, 0.05x) of anti-IgM, CpG and IFN-γ starting at 10 μg/ml, 1 μM and 50 ng/ml, respectively. During measurement, dead cells were excluded by 7-amino-actinomycin-D (7-AAD, 1 μg/ml; Thermo Fisher Scientific) and the mean fluorescence intensities of Fluo-4, corresponding to the levels of intracellular free Ca^{2+} were measured and analysed in time by flow cytometer (FACSCalibur (BD)) and CellQuest software (BD). Data were further quantified by FlowJo (FLOWJO, LLC) and the GraphPad Prism software.

2.8. Analysis of gene expression

RNA was extracted from sorted and cultured (0, 6, 24, 48 h) MZ B-cells using TRIzol Reagent (Thermo Fisher Scientific) and transcribed to cDNA by Superscript II reverse transcriptase for real-time RT-PCR analysis (Invitrogen, Thermo Fisher Scientific). Controls for genomic DNA contamination were included in the experiments. IL-10 mRNA level was quantified using primers and FAM-labelled probes from Thermo Fisher Scientific (TaqMan Real-Time PCR Assays) according to the manufacturer's instructions using the StepOnePlus™ real-time PCR system (Thermo Fisher Scientific). Variations in cDNA input were normalized against the reference gene β2 m (Thermo Fisher Scientific)

and relative changes in gene expressions were analysed by using the comparative CT method ($2^{-\Delta\Delta CT}$).

2.9. Confocal laser scanning microscopy

For the analysis of the phosphorylation and colocalization of CREB and CBP, MACS sorted MZ and FO B were stimulated with IFN-γ (50 ng/ml), anti-IgM (10 μg/ml) and CpG (1 μM) for different times (0, 1, 6, 24 h) at 37 °C and 5% CO_2. After incubation cells were washed once then fixed with Cytofix/Cytoperm solution (BD) for 20 min at 4 °C. Following two washes with Perm/Wash buffer (BD) cells were labelled with anti-mouse CBP antibody (Cell Signaling Technology) at 4 °C. After o/n incubation cells were washed twice with Perm/Wash buffer then stained with anti-rabbit-Alexa Fluor 647 conjugates (Thermo Fisher Scientific) at RT for 45 min in dark. After washes, cells were kept in the dark and labelled with Alexa Fluor 488 conjugated phospho(Ser133)-CREB-specific antibody for 60 min. Before measurement nuclei were counterstained with DAPI (1 μg/ml, Thermo Fisher Scientific) and the cells were mounted to Poly-L-Lysine coated plates (Ibidi μ-Slide). Images were taken by an Olympus FluoView 500 Laser Scanning Confocal Microscope with an 60x oil immersion objective (N.A.: 1.1). Colocalization analysis was performed > 100 cells/sample by the FluoView software. The Pearson's colocalization index was used to get estimate on the extent of protein colocalization. Pearson's colocalization index (CI): CI values close to zero indicate no or a very low degree of colocalization, CI between 0.5 and 1 reflects a high degree of colocalization, and CI = 1 value would correspond to a full overlap between the two colours in each pixel of the image.

2.10. Statistics

Statistical analyses were performed using either a nonparametric one-way or a two-way ANOVA test of the GraphPad Prism software. The applied test and p values are indicated and defined in figure legends.

3. Results and discussion

3.1. Simultaneous stimulation of the BCR, TLR9 and IFN-γ receptors induces IL-10 expression in MZ B-cells

Compared to FO B-cells, MZ B-cells have a naturally activated phenotype with a characteristically higher expression of surface IgM and even without T-cell help, following the BCR and TLR stimulation, can be readily activated to proliferate and produce different cytokines including IL-10 [21]. The simultaneous signalling initiated by the BCR and TLR9 combined with the action of IFN-γ has a pivotal role in the development and severity of systemic autoimmunity, particularly in systemic lupus erythematosus (SLE) or RA [22,23]. We have shown earlier, that these signalling cascades converging at different cellular levels, had an impact on IL-10 production and represented the least complex stimuli B-cells could get at sites of inflammation [19]. Therefore, throughout our experiments we used anti-IgM, CpG and IFN-γ simultaneously to characterize and compare the IL-10 producing capacity of MZ and FO B-cells. Intracellular flow cytometry analysis of sorted cells revealed that after an incubation period of 24 h in the presence of the indicated stimuli, FO B-cells failed to transform into IL-10 positive cells. MZ B-cells however, responded to almost all combinations of stimuli with the highest percentage of IL-10 positive cells induced by the simultaneous BCR, TLR9 and IFN-γ receptor signals (Fig. 1A). Data presented on Fig. 1B clearly show the positive effect of IFN-γ on the number of IL-10 positive MZ B-cells induced by anti-IgM and CpG. This result is in correlation with earlier findings, emphasising that IFN-γ may counteract with tissue-destroying inflammatory processes and show that IFN-γ receptor deficiency could increase the susceptibility and severity of EAE and CIA in mouse strains [24,25]. Flow cytometry data were verified by measuring the amount of the secreted cytokine by IL-10-specific ELISA in the supernatants of 24 and 48 h cultured MZ or FO B-cells (Fig. 1C, D). A relatively little amount of IL-10 was detected in the supernatants of cultured FO B-cells, probably secreted by the contaminating MZ B-cells (Suppl. Fig. 1B). However, the levels of boosted IL-10 in MZ B-cell cultures clearly show that the combined action of the BCR, TLR9 and IFN-γ receptors drives MZ B-cells to differentiate into IL-10 producing cells. Likewise, our results indicate that factors present in the microenvironment may play a crucial role in the induction of IL-10 producing cells and suggest that the two peripheral B2 B-cell subsets could behave differently under the same condition representing the sites of inflammation.

3.2. Stimulation of the BCR, TLR9 and IFN-γ receptors induces prolonged activation of CREB in MZ B-cells

Although there is little known about the combined actions of the BCR, TLR9 and IFN-γ signals in B-cells, especially in MZ B-cells, it becomes evident that the serine/threonine kinase, glycogen synthase kinase-3 (GSK-3) and CREB transcription factor are key players in integrating these responses [26,27]. GSK-3 itself is phosphorylated by serine kinase Akt and thus regulated by the PI3K-Akt pathway [28]. Activated GSK-3 phosphorylates multiple substrates, including transcription factor CREB [29]. Therefore, in our next experiments we performed Western blot analysis to follow the activation of GSK-3, Akt, CREB and p38 in time in response to simultaneous stimuli by anti-IgM, CpG and IFN-γ. Fig. 2A shows the robust phosphorylation of all the molecules at earlier time points, 30, 60 min after stimulation in both cell types. However, at around 6 h the phosphorylation levels of GSK-3, Akt, and CREB transcription factor dropped dramatically in FO B-cells, while they were sustained and decreased only by 24 h in MZ B-cells. Besides, the phosphorylation of p38, even though it was less robust in FO than in MZ B-cells, was sustained in both cell types. This result is in correlation with earlier findings that showed the synergistic effect of simultaneous stimulation of the BCR and TLR9 on MAKP kinases [30,31].The elongated activation pattern of GSK-3 and CREB in MZ B-cells *versus* shortened phosphorylation kinetics found in FO B-cells, however, reflects that GSK-3 plays an important role in balancing pro- and anti-inflammatory cytokine production, furthermore indicates the different functional responses given by the two B-cell types during inflammation [32].

3.3. Stimulation of the BCR, TLR9 and IFN-γ receptors induces robust calcium flux in MZ B-cells

BCR stimulation mediates the activation of phospholipase Cγ2 (PLCγ2), which catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate, generating diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 functions to mobilize calcium from intracellular stores, whereas DAG binds to protein kinase C [33]. Quite recently, Matsumoto and colleagues showed that ablation of the calcium (Ca^{2+}) sensors STIM1 and STIM2 in B-cells caused defects in activation of NFAT, production of anti-inflammatory cytokine, IL-10, and in suppression of an EAE model of autoimmune diseases thus provided a great evidence for the significance of STIM-dependent Ca^{2+} signalling in the regulatory function of B-cells [34]. To check the participation of Ca^{2+} in the *in vitro* regulatory differentiation of B-cells, sorted MZ and FO B-cells were loaded with Fluo-4AM and simultaneously stimulated through the BCR, TLR9 and IFN-γ receptors. Flow cytometry detection of Ca^{2+} release showed a dose-dependent calcium response in both cell types, with a clearly lower magnitude in FO B-cells (Fig. 2B, C). To quantify the data and compare cell-type- and dose-dependent responses, we calculated the Fmax/F0 ratio using the maximum fluorescence level and the initial fluorescence intensity (proportional to the resting intracellular Ca^{2+}) of the cells and presented on Fig. 2D. In accordance with the previous results, MZ B-cells responded for anti-IgM, CpG and IFN-γ stimuli with a higher increase in intracellular Ca^{2+}

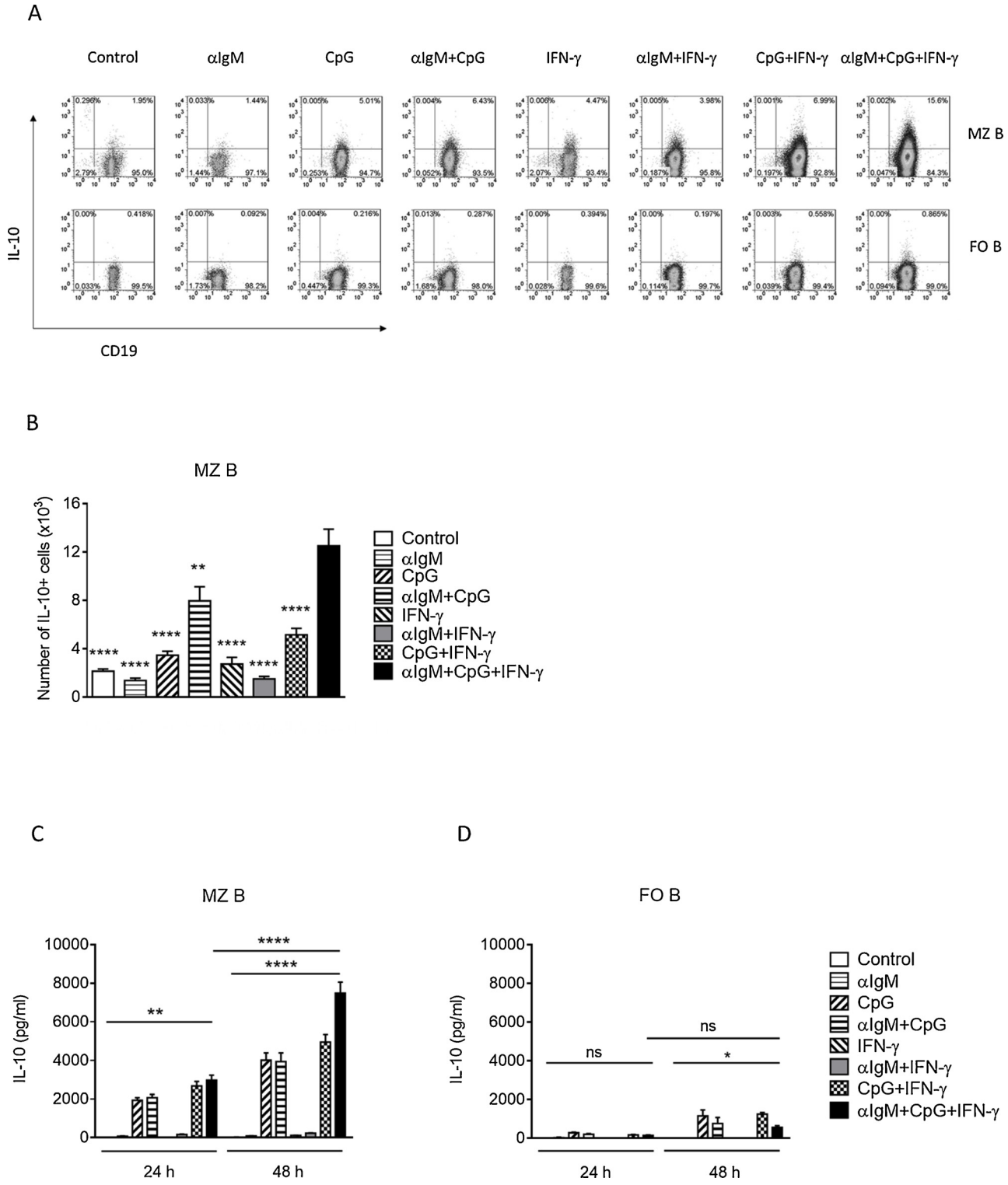

Fig. 1. Stimulation MZ B-cells through the BCR, TLR9 and IFN-γ receptors induces IL-10 expression. Sorted MZ and FO B-cells were cultured with anti-IgM, CpG and IFN-γ alone or in combination of all for 24 h. (A) Flow cytometry analysis of the intracellular expression of IL-10 in MZ or FO B-cells is shown. Percentages in the plots indicate the percent of cells falling into each gate. Control means non-stimulated but PMA, ionomycin and monensin treated cells. One representative plot is shown for all conditions. (B) Numbers of IL-10 positive MZ B-cells, defined as IL-10$^+$CD19$^+$ MZ B-cells, were calculated and shown as a mean ± SEM of six biological replicates pooled from three independent experiments. Statistical analyses were carried out using a one-way ANOVA test, significant differences were shown in comparison to the anti-IgM, CpG and IFN-γ treated sample (**$p < 0.01$, ****$p < 0.0001$). (C) Using culture supernatants, the amounts of secreted IL-10 by MZ B-cells or FO B-cells (D) were measured by ELISA after 24 or 48 h. Data are shown as the mean ± SEM of six biological replicates pooled from three independent experiments. Statistical analyses were carried out using a two-way ANOVA test, only the comparison between controls and anti-IgM, CpG and IFN-γ treated samples are shown (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$).

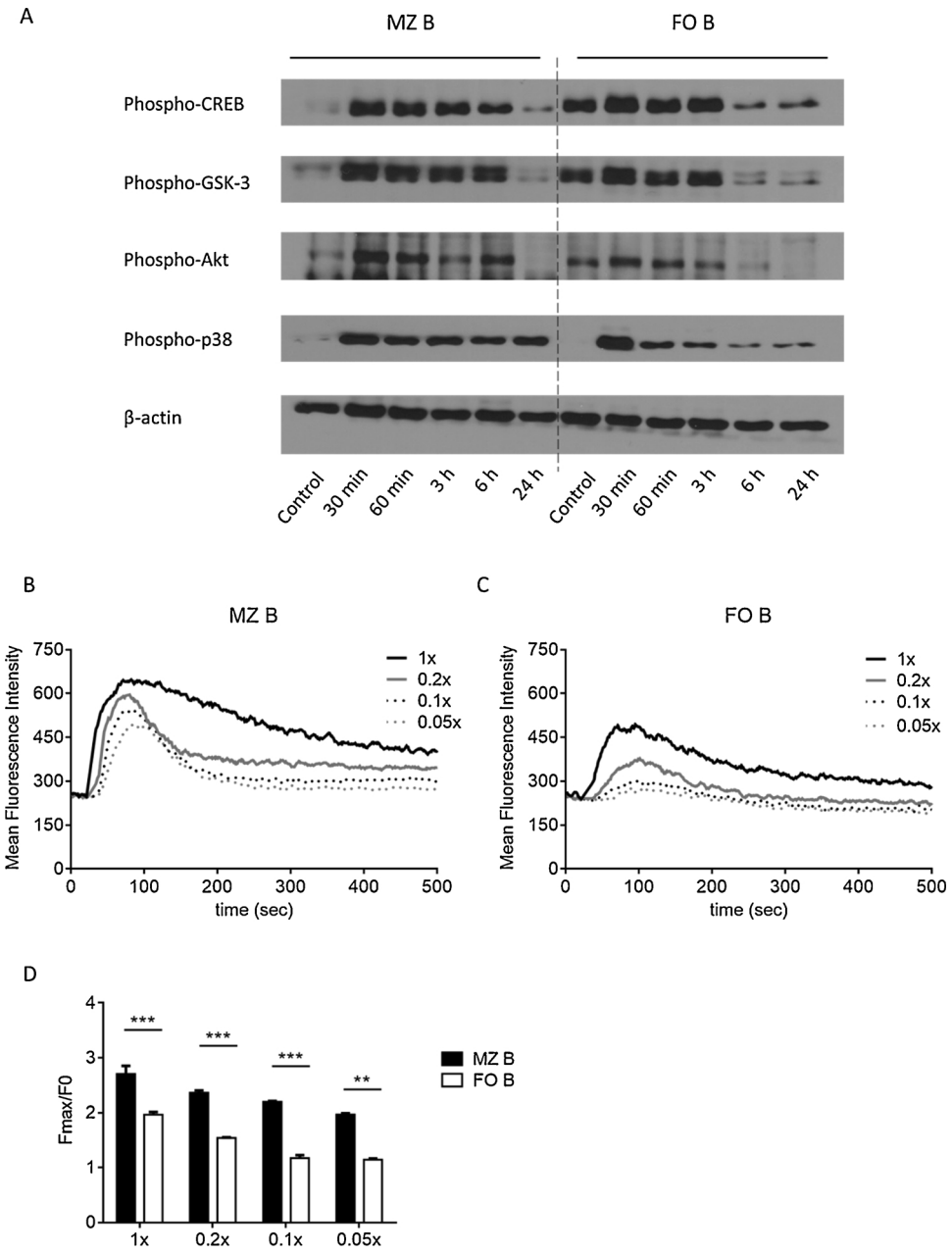

Fig. 2. Signalling characteristics induced by the BCR, TLR9 and IFN-γ receptors in MZ and FO B-cells. (A) Sorted MZ and FO B-cells were cultured in the presence of anti-IgM, CpG and IFN-γ for the indicated times (0, 30, 60 min, 3, 6, 24 h) then subjected to Western blot analysis using antibodies against the phosphorylated forms of CREB, GSK-3, Akt and p38. β-actin was used as a loading control. Data shown are from single experiments representative of three performed (Suppl. Fig. 2A, B). Intracellular Ca^{2+} response of sorted, Fluo-4AM loaded (B) MZ and (C) FO B-cells stimulated simultaneously through the BCR, TLR9 and IFN-γ receptors. Fluorescent signals induced by the decreasing concentration (0.2x, 0.1x, 0.05x) of anti-IgM, CpG and IFN-γ started at 10 μg/ml, 1 μM and 50 ng/ml, respectively, were recorded by flow cytometer. One representative example from three is shown for both cell types. (D) Quantification of the Fmax/F0 ratio using the maximum fluorescence intensity induced by the different concentrations (0.2x, 0.1x, 0.05x) of anti-IgM, CpG and IFN-γ started at 10 μg/ml, 1 μM and 50 ng/ml, respectively, and the initial fluorescence level of the corresponding sample. Data are shown as the mean ± SEM of three independent experiments. Statistical analyses were carried out using a two-way ANOVA test, significant differences are shown at each dilution (**$p < 0.01$, ***$p < 0.001$).

than FO B-cells (Fig. 2B, C). Moreover, the Fmax/F0 ratio clearly indicates that the increase in intracellular Ca^{2+} was dose-dependent and more than two fold in MZ B-cells, while a significantly lower change was mediated by every dosage of anti-IgM, CpG and IFN-γ in case of FO B-cells. These results again suggest the importance of signal strength in the regulatory differentiation of a B-cells and are in accordance with those findings concluding that the temporal profile of the Ca^{2+} signal determines the magnitude of response obtained, moreover serves as a second messenger between CREB activation and calcium responsive serine-threonine kinases [35–37].

3.4. The BCR, TLR9 and IFN-γ receptors induce prolonged binding of activated CREB to CBP in MZ B-cells

CREB is a phosphorylation-dependent transcription factor that can be induced by a variety of stimuli such as inflammatory signals and it mediates the transcription of genes containing a cAMP-responsive element (CRE). Several immune-related genes possess this CRE, including il10 [38]. Upon BCR stimulation CREB is activated by phosphorylation on Ser-133 then interacts with its coactivator protein, CREB-binding protein (CBP) to initiate transcription of CREB-responsive genes [39]. For the analysis of the binding of activated CREB to its molecular partner CBP, MACS purified MZ and FO B-cells were treated with anti-IgM, CpG and IFN-γ for the indicated times (0, 1, 6, 24 h), then harvested and stained for confocal microscopy with fluorescently labelled phosphoSer133-specific CREB and anti-CBP antibodies. While CBP was constitutively expressed in all samples, confocal images clearly show the time-dependent phosphorylation of CREB both in MZ and FO B-cells, however, with noticeable differences (Fig. 3A, B). In accordance with the Western blot data FO B-cells displayed only a short activation period peaking at 1 h after stimulation, while the phosphorylation of CREB in MZ B-cells was still detectable at 6 h. Similarly, binding of phosphorylated CREB to CBP showed the same time dependence. The two molecules had only a transient association in FO B-cells while they stayed linked much longer in MZ B-cells (Fig. 3A, B). These observable findings were quantified by calculating the colocalization index, which

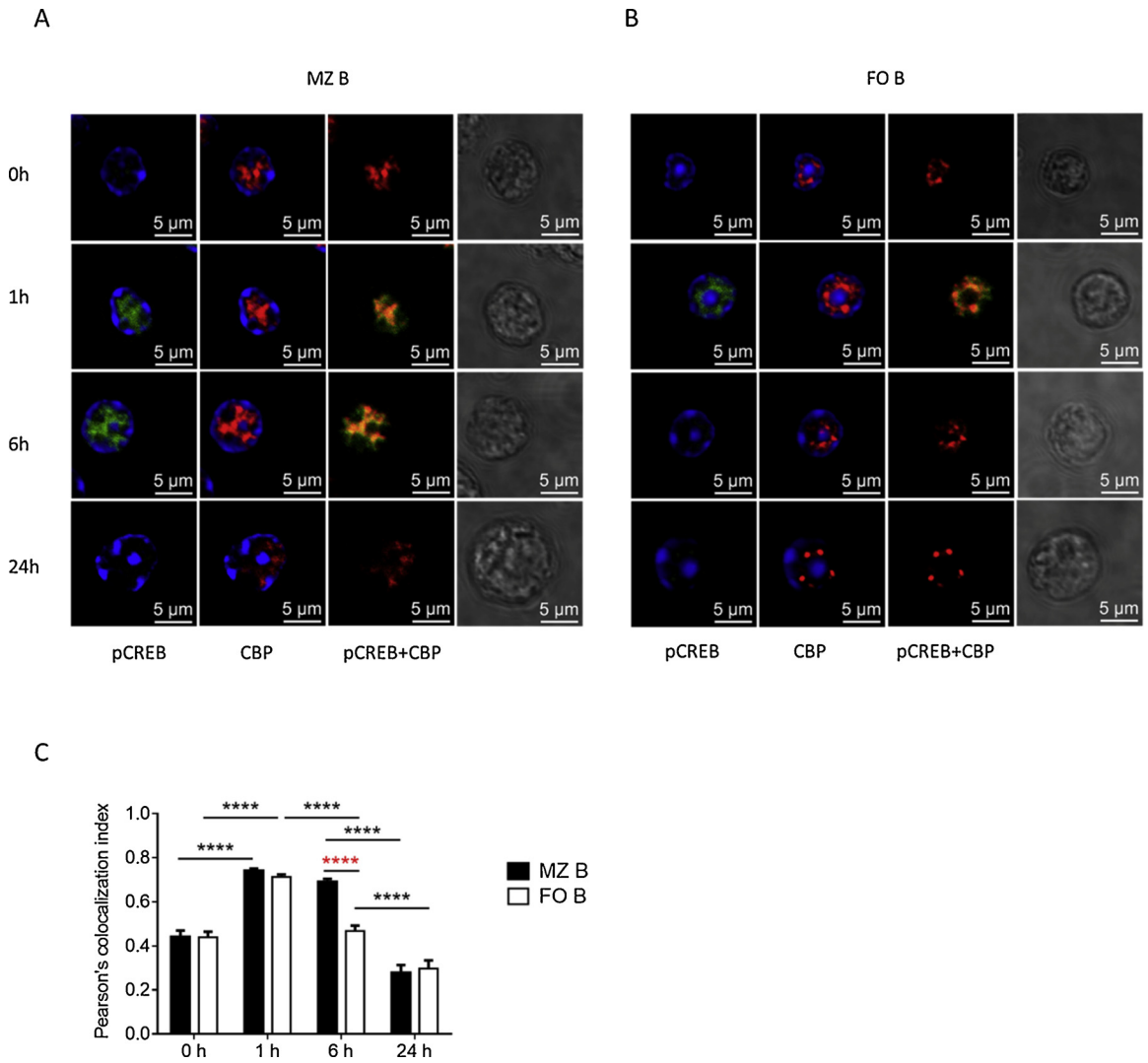

Fig. 3. The BCR, TLR9 and IFN-γ receptors induced time-dependent association of CREB and CBP. Sorted (A) MZ and (B) FO B-cells were cultured with anti-IgM, CpG and IFN-γ simultaneously for the indicated times (0, 1, 6, 24 h) then stained for fluorescent analysis. Confocal microscopic images illustrate the time-dependent phosphorylation and colocalization of CREB (green) and CBP (red). Nuclei counterstained with DAPI (blue) and DIC images (right) show cell morphology. One representative image is shown for all time points for both cell types. (C) Pearson's coefficients quantifying colocalization levels in quiescent and activated MZ (black bar) or FO (open bar) B-cells. Data are displayed as mean ± SEM of images > 100 pooled from two independent experiments. Statistical analyses were carried out using a two-way ANOVA test, significant differences are shown (****$p < 0.0001$).

evaluate the extent of spatial relationships between two molecules. Results presented on Fig. 3C show that colocalization between phospho-CREB and CBP increased significantly by 1 h in both cell types and the two molecules remained colocalized in MZ B-cells and dissociated only by 24 h. Moreover, our data prove that the colocalization between phospho-CREB and CBP differs significantly in MZ *versus* FO B-cells at the 6 h' time point and show strong correlation with the phosphorylation kinetics of CREB detected by Western blot (Fig. 2A). All these results are in line with those concepts stating that the stoichiometry of CREB phosphorylation correlates with the intensity of the stimulus and the level of target gene activation [40], moreover, support observations published by Alvarez and colleagues on dendritic cells about a CRE-dependent mechanism that regulates CREB transcriptional activity by binding the coactivator CBP and induces il10 transcription [41].

3.5. Inhibition of the binding between CREB and CBP decreases IL-10 production by MZ B-cells

CBP, acting as a cofactor and as a scaffolding protein, may increase the relative concentration of transcription factors in the local transcriptional environment and thereby facilitates the protein-protein and protein-DNA interactions [42]. Binding of activated CREB to CBP is a critical step for the transcriptional regulation of CRE-containing genes, including our gene of interest, the regulatory il-10. Therefore, blocking the interaction between CREB and CBP is a possible approach to analyse the contribution of CREB to the IL-10 production of MZ B-cells. Based on specificity, we selected a small molecule inhibitor, 2-naphthol-AS-E-phosphate (KG-501), which directly targets the KIX domain of CBP. When added to live cells, KG-501 disrupts the CREB-CBP complex and attenuates target gene induction [43].

In these experiments sorted MZ B-cells were incubated in the presence of anti-IgM, CpG, and IFN-γ w/o the CREB inhibitor and performed protein and gene expression analysis. Using IL-10-specific ELISA and the cell culture supernatants we found that the amount of secreted IL-10 decreased significantly by 48 h in the presence of the inhibitor (Fig. 4A). Similarly, measuring the levels of IL-10 mRNA expression induced by the simultaneous signals w/o the CREB inhibitor we detected a significant decrease in samples incubated with KG-501 for 48 h (Fig. 4B). The fact, that the inhibitor had no negative effect on the viability of MZ B-cells at the concentration applied, suggests that the

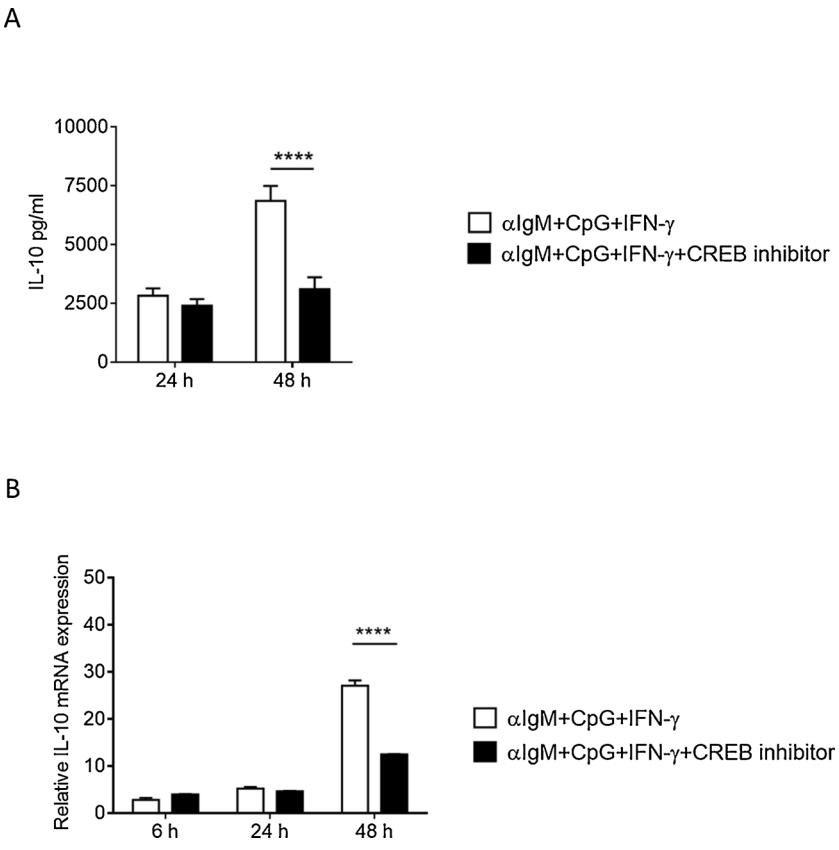

Fig. 4. Inhibition of CREB binding to CBP decreases IL-10 production by MZ B-cells. (A) Sorted MZ B-cells were cultured simultaneously in the presence of anti-IgM, CpG and IFN-γ with (black bar) or without (open bar) the addition of the CREB inhibitor for 24 and 48 h. Using culture supernatants, the amounts of secreted IL-10 by MZ B-cells were measured by ELISA. Data are shown as the mean ± SEM of five biological replicates pooled from two independent experiments. Statistical analyses were carried out using a two-way ANOVA test, significant difference is shown (****$p < 0.0001$). (B) Cultured MZ B-cells without CREB inhibitor (open bar) or in the presence of the inhibitor (black bar) were incubated for 6, 24, 48 h in the presence of anti-IgM, CpG and IFN-γ and analysed for the expression of IL-10 by real-time RT-PCR. Data were plotted relative to control, non-stimulated samples that were set as one. IL-10 mRNA expressions are shown as the mean ± SEM of four replicates pooled from two experiments, statistical analyses were carried out using a two-way ANOVA test, significant difference is shown (****$p < 0.0001$).

decrease was not the cause of the KG-501 pre-treatment itself (Suppl. Fig. 3). Although it seems interesting that there is no inhibitor-dependent decrease in IL-10 expression before 24 h, we suppose this might be due to the experimental set up. The optimal stimuli for the BCR, TLR9 and IFN-γ receptors and the applied concentration of the KG-501 inhibitor could only prevent the binding of CREB to CBP by 24 h, when the level of the phosphorylated CREB dropped significantly and inhibited further Il-10 transcription and translation between 24 and 48 h.

We believe that our results indicate the involvement of the CREB transcription factor in the IL-10 production of MZ B-cells and suggest that duration of the interaction of the CREB and CBP is a key factor in the inflammatory signal induced IL-10 secretion.

4. Conclusions

Here, we demonstrate that signalling cascades driven simultaneously by the BCR, TLR9 and IFN-γ receptors induce IL-10 secretion by MZ B-cells but leave FO B-cells unresponsive. We show that the activation of the transcription factor CREB is prolonged in MZ but transient in FO B-cells. The prolonged phosphorylation of CREB is associated with a stronger calcium signal and its sustained binding to CBP, whereas inhibition of the association between CREB and CBP decreases IL-10 secretion. Taken together we can conclude that CREB is an essential transcription factor that is involved in the IL-10 production of MZ B-cells and suggest that its prolonged activation is needed for the regulatory differentiation of B-cells under inflammatory conditions.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.06.004.

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