Molecular Immunology 62 (2014) 266–276

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Review

IL-10 production by B cells is differentially regulated by immune-mediated and infectious stimuli and requires p38 activation☆

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ARTICLE INFO

Article history:
Received 14 August 2013
Received in revised form 16 May 2014
Accepted 30 May 2014
Available online 23 June 2014

Keywords:
IL-10
B cells
CD40
TLR
MyD88
p38

ABSTRACT

IL-10 is an immune suppressive cytokine with pleiotropic effects on B cell biology. IL-10 production has a pivotal role for the regulatory suppressive functions that B cells exert in many physiological and pathological settings. Several exogenous stimuli and endogenous immune mediators can trigger IL-10-producing B cell maturation. To clarify and gain a better insight into the mechanisms of IL-10 production by B cells, we first compared the effects of LPS, CpG, agonistic CD40 mAb and BAFF on IL-10 production, and then we investigated the signal transduction mechanisms responsible for these responses. While infectious/danger stimuli determine the rapid production and release of IL-10 by B cells, a limited subset of CD40-poised, IL-10-competent B cells produce IL-10 in response to a later antigenic or infectious signal. Although BAFF is able to induce a similar subset of IL-10-competent B cells, these cells do not similarly respond to the same antigenic or infectious signals. Importantly, by using specific inhibitors of the MAP kinase pathways, we found that while *il-10* gene expression triggered by the TLR agonists LPS and CpG is strongly dependent on p38 activity, the induction of IL-10 competence in CD40-activated B cells does not depend on ERK1/2, p38 or JNK pathways.

1. Introduction

B lymphocytes are a relevant source of IL-10 and this cytokine has a pivotal role for B cell biology, as it is required for B cell survival, differentiation and isotype switching (Rousset et al., 1992; Siegel et al., 2008). Moreover the production of IL-10 has been recognized as one of the main mechanisms contributing to the immune suppressive function of B lymphocytes (Klinker and Lundy, 2012). Indeed, although B cells have been traditionally considered effector components of the immune system, in recent years a growing body of evidence has uncovered their importance in the suppressive regulation of immune responses (LeBien and Tedder, 2008). In analogy with Tregs, B cells with regulatory suppressive functions have been termed Bregs (Mizoguchi and Bhan, 2006). The main functional property common to the different Breg populations described so far is the ability to produce and secrete the immune suppressive cytokine IL-10. It has been proposed that regulatory B cells acquire the ability to produce and secrete IL-10 as the result of a stepwise process: according to this model B cells first mature a state of IL-10 competence (a suspended condition in which B cells do not actually produce IL-10) and only later are induced to produce and secrete IL-10. This later step can be mimicked *ex vivo* by a short term stimulation with LPS, PMA and ionomycin (DiLillo et al., 2010). It should be emphasized that the identification of Bregs with IL-10-producing B cells is misleading since IL-10 serves at several purposes for B cell biology and B cells are able to produce IL-10 also as the result of a process of activation and not necessarily for the purpose of regulatory functions (Conti et al., 2003).

Since B cells with regulatory functions have been discovered in autoimmunity, inflammation, infection and tolerance settings, it is likely that different stimuli both endogenous, (produced by other immune cells), and exogenous (produced during infection), can promote their expansion and/or differentiation in a context-dependent manner (Vitale et al., 2010). Indeed several endogenous mediators and infectious signals have been shown to induce IL-10 production in B cells (Mauri and Bosma, 2012), but their

Abbreviations: BAFF, B-cell activating factor; BCR, B-cell receptor; Breg, regulatory B cells; ERK, extracellular-signal-regulated kinase; JNK, c-Jun N-terminal kinase; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase; MyD88, myeloid differentiation factor 88; PMA, phorbol 12-myristate 13-acetate; TLR, toll-like receptor.

☆ This article belongs to SI: B-cell and Autoimmunity.

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http://dx.doi.org/10.1016/j.molimm.2014.05.018
0161-5890/

context-specific function in IL-10-producing B cell maturation and their interplay were poorly investigated. Moreover, little is known about the signaling pathways responsible for the differentiation and/or activation of regulatory B cells. Ca^{2+} signaling through the calcium sensors STIM-1 and STIM-2 has been shown to play an important role in IL-10 production in B cells and in the expression of their regulatory functions (Matsumoto et al., 2011). More recently, B-cell linker protein was also shown to mediate IL-10 production in regulatory B cells (Jin et al., 2013). In the human setting, it has been shown that, following CD40 activation, $CD19^{+}CD24^{hi}CD38^{hi}$ B cells from patients with systemic lupus erythematosus presented impaired IL-10 production and lower levels of STAT-3 phosphorylation, compared to the healthy counterpart (Blair et al., 2010). MAPKs were shown to play an important role in IL-10 production in different immune cell types. ERK and p38 pathways are both important for IL-10 induction in TLR-activated antigen presenting cells (Saraiva and O'Garra, 2010) while the development of IL-10-producing Th1 cells required activation of the ERK signaling cascades (Saraiva et al., 2009). Altogether these evidences prompt the investigation of the role of MAPK in IL-10 production by B cells.

In this study we compared immune-mediated and exogenous signals for their ability to trigger IL-10 competence and/or production in B cells. For this purpose, B cells were cultured with four different substances, known to promote B cell maturation and/or activation: LPS and CpG were chosen as they mimic exogenous, infectious signals, whereas BAFF and the agonistic CD40 mAb were chosen as prototypes of immune-mediated signals. The use of these substances was also due to the fact that they have in part already been related to the acquisition of the regulatory phenotype by B cells (Lampropoulou et al., 2008; Mauri et al., 2003; Yanaba et al., 2009; Yang et al., 2010). Moreover, taking advantage of specific pharmacological inhibitors, we investigated the role of MAPKs activation on the induction of IL-10-competent B cells and on the production of IL-10.

2. Materials and methods

2.1. Antibodies and chemicals

Fluorescence-conjugated mAbs to mouse CD19 (6D5) and IL-10 (JES5-16E3) were purchased from BioLegend while rat IgG2b isotype control PE was from eBioscience. The purified anti-mouse CD16/CD32 (Fc Block, 2.4G2) and anti-mouse CD40 (HM40-3) mAbs were from Becton Dickinson while the anti-mouse IgM Ab was from Jackson ImmunoResearch Laboratories. LPS, PMA, ionomycin and monensin were all purchased from Sigma-Aldrich whereas BAFF was from PeproTech and CpG from Dynavax Technologies. Inhibitors of MEK1/2 (U0126 and PD98059), p38 (SB203580) and JNK (SP600125) and the inactive analogs of SB203580 (SB202474) and of SP600125 (N^1-methyl-1,9-pyrazoloanthrone) were all purchased from Calbiochem/Merck. The p38 MAPK inhibitor VX-702, was purchased from Selleck Chemicals.

2.2. Animals, B cell preparation and culture conditions

Purified splenic B cells were obtained from 6 to 12-week-old female C57BL/6 mice (Harlan Laboratories) by a negative depletion method that removes almost all unwanted cells without manipulating the B cells themselves (Merluzzi et al., 2004). For certain experiments, B cells were obtained from spleens of *C57BL/6-Myd88*$^{-/-}$ (*Myd88*$^{-/-}$) mice, gently provided by Prof. Francesca Granucci, Department of Biotechnology and Biosciences, University of Milano-Bicocca; Milan, Italy. Purified B cells were cultured at the final concentration of 10^6 cell/ml, in the presence or absence of substances known for their ability to induce B cell maturation and/or activation. Specifically, we stimulated the cells with 1 μg/ml anti-mouse CD40 mAb, 20 ng/ml BAFF, 10 μg/ml LPS, 5 μg/ml CpG or 0.5 μg/ml anti-mouse IgM Ab.

2.3. Inhibitor assay

B cells were pre-treated for 1 h with 10, 5, 2.5 or 1.25 μM of the specific pharmacological MAPK inhibitors U0126, PD98059, SB203580 and SP600125 or of the negative analogs SB202474 and N^1-methyl-1,9-pyrazoloanthrone, before incubation with anti-mouse CD40 mAb or LPS for other 24 or 48 h. Only the 10 μM dose of these MAPK inhibitors was used in the experiments performed on CpG-stimulated B cells. Similarly, B cells were pre-treated for 1 h with 10 μM of VX-702 before incubation with LPS or CpG for other 24 or 48 h.

2.4. RNA isolation and real-time PCR

IL-10 mRNA levels were determined by real-time PCR. Total cellular RNA was isolated from 5×10^6 B cells using TRIzol reagent (Life Technologies) following manufacturer's instructions. RNA (1 μg) was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad). The generated cDNA was amplified by quantitative real-time PCR with the Bio-Rad iQ5 device and using SYBR green as detection agent (iQ SYBR Green Super Mix, Bio-Rad). Each reaction was performed in triplicate and data were collected and analyzed by the complementary computer software (CFX Manager software, Bio-Rad). Results were expressed as fold induction compared to the control condition. The G3PDH transcript levels were used to normalize samples. The primers used for the real-time PCR reactions were synthesized and purified by Eurofins MWG Operon and had the following sequences: murine *il-10* (forward 5′-TGTGAAAATAAGAGCAAGGCAGTG-3′; reverse 5′-CATTCATGGCCTTGTAGACACC-3′), murine *g3pdh* (forward 5′-TCAACAGCAACTCCCACTCTTCCA-3′; reverse 5′-ACCCTGTTGCTGTAGCCGTATTCA-3′).

2.5. Detection of intracellular and secreted IL-10

For immunofluorescent staining of intracellular IL-10, $1–2 \times 10^6$ B cells were resuspended (1×10^6 cells/ml) in culture medium alone or containing 50 ng/ml PMA, 500 ng/ml ionomycin, 10 μg/ml LPS and 2 μM monensin and cultured for 5 h at 37 °C and 5% CO_2. The protocol for intracellular staining was as described in Yanaba et al. (2009). Stained samples were acquired on FACScan (Becton Dickinson) while flow cytometry data were analyzed with FlowJo software (Tree Star).

The levels of IL-10 in cell supernatants were quantified by ELISA. 0.6×10^6 B cells were cultured in the presence or absence of stimulation for 48 h, at the final concentration of 10^6 cell/ml in a 24-well flat bottom plate (Corning costar). The mouse IL-10 ELISA Ready-SET-Go kit (eBioscience) was used, following manufacturer's instructions.

2.6. Cell viability assay

To test the effect of the MAPK inhibitors on cell viability, B cells were stimulated either with anti-mouse CD40 mAb or LPS, in the presence or absence of the aforementioned MAPK inhibitors. After 48 h of culture, necrotic and apoptotic cells were detected by staining with annexin V and propidium iodide. For this purpose, the Annexin V-FITC apoptosis detection kit (eBioscience) was used following manufacturer's instructions. Stained samples were acquired on FACScan and flow cytometry data were analyzed with FlowJo software.