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Modulation of CD40-activated B lymphocytes by N-acetylcysteine involves decreased phosphorylation of STAT3

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ABSTRACT

B lymphocyte activation, maturation and reshaping require the interaction of its receptor CD40 with its ligand CD154, which is expressed on activated T lymphocytes. Metabolism in activated B lymphocytes is also characterized with several REDOX changes including fluctuation of Reactive Oxygen Species (ROS). Herein, we first confirm that stimulation of human peripheral blood B lymphocyte with CD154 increases intracellular ROS level. Then, by treatments with two well-known antioxidants, N-acetylcysteine (NAC) and Trolox, we further investigate the influence of REDOX fluctuation in CD40-activated B lymphocyte homeostasis in long term culture (13 days). Treatments with NAC increase viability, decrease proliferation and Ig secretion and enhance homoaggregation of B lymphocytes while Trolox only induces a marginal increase of their Ig secretion. The NAC-induced homoaggregation phenotype is paralleled with increased expressions of CD54, CD11a, CD27 and CD38. Mechanistically, a 24 h exposure of B lymphocytes with NAC is sufficient to show strong inhibition of STAT3 phosphorylation. Besides, the treatment of B lymphocytes with the STAT3 inhibitor VI increases viability and decreases proliferation and secretion as in NAC-treated cells thus showing a role for STAT3 in these NAC-induced phenotypes. This study done in a human-based model provides new findings on how REDOX fluctuations may modulate CD40-activated B lymphocytes during immune response and provide additional hints on NAC its immunomodulatory functions.

1. Introduction

Interactions between CD40$^+$ B lymphocytes and CD154$^+$ T lymphocytes are essential for primary and secondary immune responses. The stimulation of the TNF receptor family member CD40 by its ligand CD154, being expressed on T lymphocytes only when activated, is required for B cell stimulation and formation of germinal center. As CD154 binds CD40, the trimerization of the receptor promotes the recruitment of adaptor proteins such as TRAFs (Tumor Necrosis Factor Receptor associated factors) or JAK3 (Janus Kinase 3), which will then regulate Mitogen-Activated Protein Kinase (MAPK), NF-κB, STATs and Src signaling pathways (Cayer et al., 2009; Néron et al., 2006b) and reviewed in (Bishop et al., 2007; D'Orlando et al., 2007; Elgueta et al., 2009). This, combined with B lymphocyte stimulation with other co-stimuli such as cytokines, drives B lymphocytes into various cellular processes such as proliferation, differentiation and Ig secretion (reviewed in (Quezada et al., 2004; van Kooten and Banchereau, 2000)). Currently, several in vitro models reproducing CD40–CD154 interaction are used to investigate physiological functions of human B lymphocytes (Neron et al., 2011). Our work on human B lymphocytes is indeed based on CD40-activation using a fibroblast cell line, which constitutively express CD154$^+$ (Néron et al., 1996). This in vitro model is effective to reproduce the outcome of human B lymphocytes within the germinal center namely, proliferation, isotype switching, as well as differentiation into immunoglobulin secreting cells (Fecteau and Neron, 2003; Fecteau et al., 2009; Néron et al., 2005).

In recent years, many studies underlined the role of REDOX homeostasis in a great variety of cellular processes including regulation of immune response (Guzik et al., 2003; Hultqvist et al., 2009; Reth, 2002). To fight infections, phagocyte cells are able to destroy infectious agent by producing, via the activation of the multicomponent enzyme NADPH oxidase, toxic concentrations of Reactive Oxygen Species (ROS) (Valko et al., 2007). Although deleterious in this previous case, ROS in adequate concentrations have been proven essential as they regulate several signaling pathways and thus cellular processes such as proliferation, differentiation, programmed cell death and survival (reviewed in (Groeger et al.,

Abbreviations: ERK, extra-signal-regulated kinases; FCB, fluorescent cell barcoding; JAK3, Janus Kinase 3; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MFI, median fluorescence intensity; NAC, N-acetylcysteine; ROS, Reactive Oxygen Species; STAT3, signal transducer and activator of transcription 3; TRAFs, tumor necrosis factor receptor associated factors.

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2009; Stone and Yang, 2006; Winterbourn, 2008)). As for B lymphocytes, ROS and generally REDOX homeostasis have also been reported to play a role in their activation, maturation and reshaping (Masciarelli and Sitia, 2008). Indeed, in human B cell lines representing different stages of B lymphocyte differentiation, Nilsson et al. reported that the expression of thioredoxin and protein disulfide isomerase, two protein associated with REDOX regulation, was different whether a cell line was representing an immature or plasma cell-like B cell (Nilsson et al., 2004). Furthermore, exposure of human B cell line Ramos and BJAB to ROS and NADPH oxidase-activating reagents upregulates tyrosine phosphorylation suggesting a role for ROS in signaling inside B lymphocytes (Suzuki and Ono, 1999). In the same line of evidences, ROS are produced following B cell receptor activation (Capasso et al., 2010) and treatment of B lymphocytes with hydrogen peroxide mimics the signal engaged by the antigen-driven activation of the BCR (Reth, 2002) suggesting that ROS are important second messenger in B lymphocyte activation. In fact, multiple myeloma (a plasma cell-associated malignancy) has been associated with deregulated oxidative stress and a decrease in antioxidant capacity (Sharma et al., 2009). In mouse B splenocytes, it has been shown that their LPS-driven transition to plasma cell requires both upregulation of ROS as well as antioxidants, which are believed to cope with the concomitant increase of oxidants (Bertolotti et al., 2010; Vene et al., 2010). Also in LPS-treated mouse B splenocytes, Moon et al. demonstrate that an increase in ROS production regulates the expression of the B-cell activating factor (BAFF) (Moon et al., 2006).

Studies have also demonstrated that ROS production occurs after CD40 activation. Indeed, ROS production following CD40 cross-linking has been associated to the activation of NADPH oxidase in mouse WEHI 231 B cell (Ha and Lee, 2004) and in human umbilical vein endothelial cell (Xia et al., 2010) but also through 5-Lipooxygensase activation in human B cell line Raji (Ha et al., 2011). The treatment of CD40-activated mouse splenocytes or mouse B cell lines with antioxidants downregulated several signaling pathway such as JNK, p38, AKT and NF-κB (Lee, 2003; Lee et al., 2007) and inhibited secretion of interleukin-6 (Lee and Koretzky, 1998). Besides, studies done with mouse splenocytes as well as the human B cell line Ramos showed that APE/Ref-1, a REDOX-dependent transcription factor, was downstream of CD40 signaling (Merluzzi et al., 2004, 2008). Most of these studies have been done in model based on murine B lymphocytes or murine and human B cell lines. Nevertheless, they provide great evidences on the relation between CD40 activation and the role of ROS in its downstream signalization.

In this study, our experimental model is based on human B lymphocytes isolated from peripheral blood and activated in vitro through CD40 using a fibroblast cell line, which constitutively expresses CD154 (Néron et al., 1996). This model is effective to reproduce the outcome of human B lymphocytes within the germinal center namely, proliferation, isotype switching, as well as differentiation into immunoglobulin secreting cells (Fecteau and Neron, 2003; Fecteau et al., 2009; Néron et al., 2005). This interaction between B lymphocytes and the fibroblast cell line also mimics more accurately the immunological synapse formed between B and T lymphocytes in the germinal center (Yuseff et al., 2009). Here, we report how N-acetylcysteine (NAC) and Trolox, two-well known antioxidants, may regulate homeostasis of CD40-activated B lymphocytes either by ROS fluctuation or by signal transduction.

2. Materials and methods

2.1. Isolation of human peripheral B lymphocytes and culture

This study has been approved by Hema-Quebec's Research Ethics Committee. Samples were collected from healthy individuals after their signature of an informed consent. Human peripheral blood leukocytes were recovered from leucoreduction systems and Peripheral Blood Mononuclear Cell (PBMC) were isolated by centrifugation using Ficoll-Plaque (GE Healthcare, Piscataway, USA), as previously described (Néron et al., 2006a, 2007). Afterwards, B lymphocytes were negatively isolated from PBMC with the EasySep Human B cell enrichment kit according to the manufacturer's instructions (StemCell Technologies, Vancouver, Canada). For culture, B lymphocytes were seeded in 6-well plates on a layer of gamma-irradiated mouse L929 fibroblasts stably expressing CD154, the L4.5 cells or when indicated mock-transfected L929 cells (Néron et al., 1996). Culture medium consisted of Iscoves's modified Dulbecco's medium (IMDM) supplemented with 10% Ultra-low IgG fetal bovine serum (Invitrogen, Burlington, Canada) also containing 50 U mL^{-1} IL-2, 20 U mL^{-1} IL-10 (Peprotech, Rocky Hil, NJ, USA), 100 U mL^{-1} IL-4 (R&D Systems, Minneapolis, MN, USA), 10 μg mL^{-1} insulin, 5.5 μg mL^{-1} transferin, 6.7 ng mL^{-1} sodium selenite (all from Invitrogen), 100 μg mL^{-1} Penecillin-Streptomycin and, for specific experiments, 0.5–10 mM N-acetyl-L-cysteine (NAC) (Sigma-Aldrich, Oakville, ON, Canada), 50 μM Trolox or 30 μM of STAT3 inhibitor VI S3I-201 (EMD Biosciences, San Diego, CA, USA). The L4.5/B cell ratio was maintained at 1:4. As determined by quantification of CD154 expression on L4.5 (Néron et al., 2005), this ratio approximately correspond to 2000–3000 CD154 molecules for each B lymphocyte. Every 2–3 days, half of the medium was renewed with appropriate reagents while L4.5 cells were renewed and B lymphocytes used for tests at days 4, 8 and 13 of culture (see Suppl. Fig. 1 for an illustration of typical B lymphocyte culture).

Cell cultures were maintained in a humidified 10%-CO_2 atmosphere at 37 °C. Cell counts and viability were determined in triplicate by Trypan blue dye exclusion. When indicated, cell phenotypes were monitored with a Nikon Eclipse TE2000 microscope equipped with 10× 0.25 NA objective lens. Images were captured using a QImaging Retiga 1300 camera and SimplePCI acquisition software (Compix Inc., Cranberry, PA, USA).

2.2. Flow cytometry analysis for surface proteins

Fluorescein isothiocyanate (FITC)-conjugated anti-CD11a and anti-CD27 as well as allophycocyanin (APC)- conjugated anti-CD19 and anti-CD54 were obtained from BD Biosciences (San Jose, CA, USA), APC-efluor 780-conjugated anti-CD19, APC-conjugated anti-CD38, and phycoerythrin (PE)-Cy7-conjugated anti-CD45 were from eBiosciences (San Diego, CA, USA) and PE-conjugated anti-CD138 from Abcam (Cambridge, MA, USA). All Abs were IgG_1 mouse monoclonal antibodies. In all assays, dead cells were delineated using Pacific Blue succinimidyl ester following manufacturer's instructions (Invitrogen). Cells were then stained, fixed with 2% paraformaldehyde (PFA), washed and suspended in 1 mL PBS containing 1% FBS and 0.01% NaN_3.

2.3. ROS detection

Reactive Oxygen Species (ROS) detection was performed in cells prepared in Hanks' balanced salts buffered solution (HBSS) (Sigma-Aldrich). B lymphocytes were stained with APC-anti-CD19. When thawed before analyses, cells were left overnight in IMDM containing 10% FBS. All viable cells were previously probed as above with Pacific Blue, then washed with HBSS and incubated with 1 μM of 5-(and-6-)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA) (Invitrogen) for 15 min.

2.4. Fluorescent cell barcoding

The determination of protein phosphorylation was done using Phospho-Flow and Fluorescent Cell Barcoding (FCB) techniques as